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PEROXISOMES IN BROWN TROUT (*Salmo trutta* f. *fario*): REGULATION BY ESTROGENS

Dissertação de Candidatura ao grau de Doutor em Ciências Biomédicas submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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À minha mãe,

por tudo…

DIRECTIVAS LEGAIS

No cumprimento do disposto no nº 2 do Artigo 8º do Decreto-Lei nº388/70 (Decreto-Lei nº 216/92 de 13 de Outubro), declara-se que a autora desta dissertação participou na concepção e na execução do trabalho experimental que esteve na origem dos resultados apresentados, bem como na sua interpretação e na redacção dos respectivos manuscritos.

Nesta tese inclui-se um artigo científico publicado numa revista internacional resultante de uma parte dos resultados obtidos no trabalho experimental, referenciado como:

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CONTENTS

ABSTRACT

Although the importance of peroxisomes in plants, fungi and protozoa has been recognized for long, the recognition of their relevance in mammal cellular metabolism was essentially based on the discovery of a class of severe human inherited diseases caused by peroxisomal deficiencies. Also the observation that many pharmaceutical and industrial compounds are able to induce proliferation of these organelles, and that prolonged treatment of rodents with most peroxisome proliferators origins hepatic tumours has been attracting researchers. On the other hand, recent evidence on the relationship between environmental pollutants and peroxisome proliferation phenomena is raising growing interest in the study of these organelles in aquatic organisms. Nonetheless, the information available on fish peroxisomes and the related PPARs (peroxisome proliferator activated receptors) is, so far, scarce. As female brown trout (*Salmo trutta* f. *fario*) has been shown to undergo hepatic peroxisome related seasonal variations, the purpose of this thesis was to provide a better understanding of the hypothesized regulation mechanisms of fish peroxisomes by estrogenic compounds, including initial approaches for studying the involvement of PPARs and estrogen receptor in the process. This work comprises a molecular component, involving the study of PPARs and peroxisomal enzymes genes, and a morphological and biochemical component, targeting peroxisomal morphofunctional parameters.

All PPAR isotypes genes were identified in brown trout through partial sequenciation and comparison with other species isotypes. For the first time, a parallel semi-quantitative and quantitative study of PPARs mRNAs was made in fish, showing the relative expression of these receptors in several organs and also their hepatic expression along the year in both genders. Among the organs tested, PPARα was more expressed in white muscle, heart and liver. PPARβ, the most strongly expressed isotype, was particularly abundant in testis, heart, liver, white muscle and trunk kidney. With a much weaker expression, PPARγ mRNA was only detected in trunk kidney, liver and spleen. PPARα expression in females was higher in early vitellogenesis and lower in late vitellogenesis than in all other seasons. In early vitellogenesis, its expression was higher for females than for males. PPARβ expression in males was higher in prespawning than in the other seasons. PPARγ was more expressed in postspawning than in late vitellogenesis

and prespawning, for females. As to males, its expression was higher in postspawning than in all the other seasons.

The gene encoding for catalase was identified in the same way and its organ distribution pattern established by real-time RT-PCR, as well as its seasonal hepatic expression in both genders. It was more expressed in liver and blood, followed by testis, white muscle and trunk kidney. In females, hepatic catalase expression was higher in postspawning and early vitellogenesis than in late vitellogenesis and prespawning. Concerning gender differences, higher levels of expression were observed for males in prespawning.

The kinetics of morphological and metabolic alterations induced by waterborne estradiol in hepatic peroxisomes was followed within 30 days of exposure and 15 days after cessation of hormone treatment. Both catalase and urate oxidase activities were negatively influenced by estradiol, although exhibiting distinct behaviours. Catalase responded in a faster way to the treatment and to its suspension, and partially recovered its activity with a low dosage of the estrogen receptor inhibitor ICI 182,780 in the water (ICI:estradiol ratio of 1:9 in molarity). Urate oxidase showed a slower response in both cases and its activity was not affected by the estrogen receptor inhibitor under these conditions. Variations on peroxisome morphology under the same circumstances were less pronounced. Only the relative peroxisome volumes were negatively affected and just by the end of treatment. Likewise, waterborne ICI in this concentration did not have a significant effect on these parameters.

Both PPARα and catalase expressions in female brown trout liver followed the same annual variation patterns as morphological and biochemical peroxisomal parameters, as well as an inverse pattern relatively to plasma estradiol levels previously determined. The effect of estradiol supply for a month on these peroxisomal enzymes of juveniles was similar to the effect of endogenous hormones on the same enzymes of late vitellogenic and prespawning mature females. These findings supported the baseline hypothesis that trout hepatic peroxisomes are directly or indirectly regulated by a mechanism involving estradiol and further suggest that the estrogen receptor and PPARα play a role in the process. Seasonal patterns of PPARs expression were shown herein for the first time in fish, opening hypotheses for research on their endogenous regulation.

RESUMO

Embora a importância dos peroxisomas nas plantas, nos fungos e nos protozoários tenha sido desde cedo reconhecida, o reconhecimento da sua relevância no metabolismo celular dos mamíferos deveu-se, sobretudo, à descoberta de uma classe de doenças humanas hereditárias severas causadas por deficiências a nível peroxisomal. Também a constatação de que muitos fármacos e compostos industriais são capazes de induzir a proliferação destes organelos, e de que o tratamento prolongado de roedores com a maioria dos proliferadores peroxissomais origina tumores hepáticos malignos tem atraído investigadores. Por outro lado, o estudo de peroxissomas de animais aquáticos tem merecido maior atenção nos últimos anos devido às evidências que apontam para uma relação entre os poluentes ambientais e os fenómenos de proliferação peroxissomal. No entanto, a informação existente sobre peroxissomas e PPARs (receptores activados por proliferadores peroxissomais) de peixes é ainda limitada. Na sequência de estudos anteriores, que mostraram que as fêmeas de truta fário (*Salmo trutta* f. *fario*) sofrem alterações sazonais nos peroxissomas hepáticos, o objectivo desta tese foi contribuir para uma melhor compreensão dos hipotéticos mecanismos de regulação dos peroxissomas de peixes por compostos estrogénicos, incluindo uma primeira aproximação ao estudo do envolvimento dos PPARs e do receptor de estrogénios no processo. Este trabalho compreende uma componente molecular, abrangendo um estudo genético dos PPARs e de enzimas peroxissomais, e uma componente morfológica e bioquímica, direccionada para parâmetros morfofuncionais dos peroxisomas.

Os genes dos três isotipos de PPARs foram identificados na truta fário por sequenciação parcial e comparação com os isotipos de outras espécies. Foi, pela primeira vez, efectuado em peixes um estudo simultaneamente semi-quantitativo e quantitativo de mRNAs de PPARs, revelando a expressão relativa destes receptores em vários órgãos e ainda a sua expressão hepática ao longo do ano em ambos os sexos. Entre os órgãos analisados, o PPARα mostrou maior expressão no músculo branco, coração e fígado. O PPARβ, o isotipo com maior expressão, revelou-se particularmente abundante no testículo, coração, fígado, músculo branco e rim posterior. Com uma expressão bastante mais fraca, o mRNA do PPARγ foi apenas detectado no rim posterior, fígado e baço. A expressão do PPARα nas fêmeas foi mais elevada durante o início da vitelogénese

e mais baixa durante a vitelogénese avançada do que nas outras épocas. No início da vitelogénese, a sua expressão foi maior nas fêmeas do que nos machos. A expressão do PPARβ nos machos foi mais elevada na pré-postura do que em todas as outras estações. Nas fêmeas, o PPARγ teve maior expressão durante a pós-postura do que durante a vitelogénese avançada e a pré-postura. Em relação aos machos, a sua expressão mostrou-se mais elevada na pós-postura do que em todas as outras estações.

O gene da catalase foi igualmente identificado e o seu padrão de expressão nos órgãos estabelecido através de RT-PCR em tempo real, assim como a sua expressão hepática sazonal em ambos os sexos. A sua expressão revelou-se mais elevada no fígado e no sangue, seguida pelo testículo, músculo branco e rim posterior. Nas fêmeas, a expressão hepática da catalase foi mais elevada na pós-postura e no início da vitelogénese do que na vitelogénese avançada e na pré-postura. Diferenças entre sexos foram notadas durante a pré-postura, com níveis mais elevados para os machos.

A cinética das alterações morfológicas e metabólicas induzidas por estradiol exógeno nos peroxissomas hepáticos foi seguida durante 30 dias de exposição à água contendo a hormona e ainda 15 dias após a suspensão do tratamento. A catalase e a urato oxidase mostraram-se negativamente influenciadas pelo estradiol, embora exibindo comportamentos distintos. A catalase reagiu de modo mais rápido ao tratamento, bem como à sua suspensão, tendo recuperado parcialmente a sua actividade com a aplicação de uma concentração baixa do inibidor do receptor de estrogénios ICI 182,780 na água (razão ICI:estradiol de 1:9 em molaridade). A urato oxidase mostrou uma resposta mais lenta em ambos os casos e a sua actividade não foi afectada pelo inibidor, nestas condições. Nas mesmas condições, as variações na morfologia dos peroxissomas foram menos pronunciadas. Apenas os volumes peroxissomais relativos foram negativamente afectados e só no fim do tratamento. Do mesmo modo, o inibidor administrado na água nesta concentração não exerceu um efeito notório nestes parâmetros.

A expressão do PPARα e da catalase hepáticos nas fêmeas de truta fário seguiram os mesmos padrões de variação anual do que alguns parâmetros peroxissomais morfológicos e bioquímicos, bem como um padrão inverso ao dos níveis plasmáticos de estradiol previamente determinados. O efeito do fornecimento de estradiol durante um mês nestas enzimas peroxissomas de juvenis foi semelhante ao efeito das hormonas endógenas nas mesmas enzimas que ocorre

nas fêmeas em vitelogénese avançada e em pré-postura. Estas descobertas apoiam a hipótese inicial de que os peroxissomas de truta são regulados directa- ou indirectamente por um mecanismo envolvendo estradiol e sugerem ainda que o receptor de estrogénios e o PPARα desempenham um papel no processo. Os padrões sazonais da expressão dos PPARs foram aqui descritos pela primeira vez em peixes, deixando em aberto a hipótese de investigação sobre a sua regulação endógena.

RESUME

Bien que l'importance des peroxisomas dans les plantes, les fongus et les protozoaires a été depuis tôt reconnue, la reconnaissance de son importance dans le métabolisme cellulaire des mammifères se soit due, surtout, à la découverte d'une classe de maladies humaines héréditaires sévères causées par des insuffisances à niveau peroxysomal. Aussi la constatation que beaucoup de médicaments et composés industriels sont capables d'induire la prolifération de ces organites, et que le traitement prolongé des rongeurs avec la majorité de proliférateurs des peroxysomes donne lieu à des tumeurs hépatiques malignes a attiré les investigateurs. Par l'autre sens, l'étude des peroxysomes chez les animaux aquatiques mérite une plus forte attention ces derniers années, surtout, dû au fait des évidences suggérant un rapport entre les polluants environnementaux et la prolifération peroxysomal. Cependant, l'information sur les peroxysomes et les PPARs (récepteurs activés par les proliférateurs des peroxysomes) chez les poissons est encore limitée. A la suite des études précédentes, montrant que chez les femelles de la truite fario (*Salmo trutta* f. *fario*) les peroxysomes hépatiques souffrent des variations saisonnières, l´objectif de cette étude était de meilleur comprendre les mécanismes de l'hypothétique régulation des peroxysomes par les composés œstrogéniques chez les poissons, y compris une première approches à l'étude de l'engagement des PPARs et du récepteur de l'œstrogène dans ce processus. Ce travail comprend un abordage moléculaire par une étude génétique des PPARs et des enzymes peroxysomales et un abordage morphologique et biochimique vers les paramètres morpho-fonctionnels des peroxysomes.

Les gènes des trois isotypes de PPARs furent identifies chez la truite fario par une séquenciation partielle et comparaison avec les isotypes d'autres espèces. Une étude semi-quantitatif et quantitatif simultané des mRNAs des PPARs montrant l'expression relative de ces récepteurs dans plusieurs organes et aussi leur expression hépatique au cours de l'année chez les deux sexes, a été réalisée par la première fois chez les poissons. Parmi les organes analysés, le PPARα a montré une plus forte expression dans le muscle blanc, le coeur et le foie. Le PPARβ, isotype le plus exprimé, c'est montré particulièrement abondant dans le testicule, le coeur, le foie, le muscle blanc et le rein postérieur. Montrant une expression beaucoup plus faible, le mRNA du PPARγ était présent jusque dans le rein

postérieur, le foie et la rate. L'expression du PPARα chez les femelles était plus élevée pendant la pré-vitellogenèse et plus faible pendant la vitellogenèse avancée que dans les autres périodes. Pendant la pré-vitellogenèse son expression était plus forte chez les femelles que chez les mâles. L'expression du PPARβ chez les mâles était plus forte pendant la pré-ponte que ailleurs. Chez les femelles le PPARγ était plus fort pendant la post-ponte que pendant la vitellogenèse avancée ou la pré-ponte. En ce qui concerne les mâles son expression c'est révélé plus forte pendant la post-ponte que ailleurs.

Le gène de la catalase fût aussi identifié et son sa forme d'expression dans les organes établi à partir de RT-PCR en temps réel, aussi bien que l'expression hépatique saisonnière chez les deux genres. Leur expression était plus forte dans le foie et le sang, moins dans le testicule, muscle blanc et rein postérieur. Chez les femelles l'expression hépatique de la catalase était plus forte dans la post-ponte et pré-vitellogenèse que dans la vitellogenèse avancée et la pré-ponte. Des différences entre genres furent observées pendant la pré-ponte, aux niveaux plus élevés chez les mâles.

La cinétique des modifications morphologiques et métaboliques induites par l'estradiol exogène dans les peroxysomes hépatiques fût suivie pendant les 30 jours d'exposition à l'eau contenant l'hormone et aussi pendant 15 jours après l'arrêt du traitement. Les activités de la catalase et de l'urato oxydase étaient négativement influées par l'estradiol, mais à des comportements distingués. La catalase a réagit de façon plus rapide au traitement et aussi à son stoppage, ayant récupéré partiellement son activité lorsqu'on applique une petite concentration de l'inhibiteur du récepteur d'estrogènes ICI 182,780 dans l'eau (à une raison de ICI:estradiol de 1:9 en molarité). L'urato oxydase a montré une réponse plus lente dans les deux cas et son activité n'a pas été affectée par le ICI, dans ces conditions. Les variations morphologiques des peroxysomes étaient beaucoup moins évidentes, dans ces mêmes conditions. Les volumes peroxymales relatifs furent les seuls négativement affectés et rien qu'à la fin du traitement. De même, le ICI ajouté à l'eau dans cette concentration n'a pas exercé un effet évident dans ces paramètres.

Les modèles de variations annuelles définies pour l'expression du PPARα et de la catalase hépatiques chez les femelles de la truite fario montrent une relation positive avec les paramètres peroxymales morphologiques et biochimiques, aussi bien qu'une relation négative avec les niveaux plasmatiques de l'estradiol

déterminés auparavant. L'effet de fournir l'estradiol pendant un mois à ces enzymes peroxymales des juvéniles fût ressemblant à l'effet des hormones endogènes dans les mêmes enzymes observable chez les femelles en vitellogenèse avancée et en pré-ponte. Ces découvertes soutiennent l'hypothèse initiale dont les peroxysomes de la truite sont régulés direct ou indirectement par un mécanisme impliquant l'estradiol et suggèrent aussi que le récepteur des estrogènes et le PPARα jouent un rôle dans le processus. Les normes saisonnières de l'expression des PPARs ont été décrites ici pour la première fois sur les poissons, en laissant en ouvert l'hypothèse de recherche sur leur règlement endogène.

ABBREVIATIONS

cDNA – complementary DNA

CoA – coenzyme A

DAB – 3,3'-diaminobenzidine

DNA – deoxyribonucleic acid

DNase – deoxyribonuclease

dNTP – deoxyribonucleotide

ELISA – enzime-lynked immunosorbent assay

FAD – flavin adenine dinucleotide

LBD – ligand binding domain

mRNA – messenger RNA

PCR – polymerase chain reaction

PPAR – peroxisome proliferator activated receptor

PPRE – peroxisome proliferator response element

RNA – ribonucleic acid

ROS – reactive oxygen species

RT-PCR – reverse transcription PCR

RXR – retinoid X receptor

SD – standard deviation

Tris-HCl – tris (hydroxymethyl) aminomethane hydrochloride

1. General Introduction

1.1. The brown trout

1.1.1. Life cycle

Depending on their natural habitat and life cycle, *Salmo trutta* species can be found in distinct populations: the migratory trout, *Salmo trutta* morpha *trutta* or sea trout, which divides the life period between rivers and seas, and the freshwater trout or brown trout, which comprises *Salmo trutta* morpha *fario* and *Salmo trutta* morpha *lacustris*, and spend all their lives in rivers and lakes, respectively (Elliott, 1994; Watson, 1999). Brown trout is widely distributed across the world, especially in Europe, from where it derived. It is also known as spotted trout because of its little coloured spots. The brown trout specimens used in this study derived from the Portuguese freshwater resident *Salmo trutta* morpha *fario* (or *Salmo trutta* f. *fario*) population, inhabiting the north region of the country. Its main economic interest is related to tourism, especially recreational fishing, and its culture has been assumed essential to the conservation of wild populations (Laikre *et al.*, 1999).

Brown trout prefer cool, unpolluted and well oxygenated streams with a swift flow in a stone and gravel bottom. In proper growth conditions, which include low population density, good food availability and an optimal temperature range from 13º to 18º C, they grow continuously with age. Depending on the habitat, a 4-year-old animal may weigh from 20 g to 1 Kg. Although they can live up to about 20 years, most of the specimens dye much younger (Reviewed by Klemetsen *et al.*, 2003).

These trouts became sexually mature at an age between 1 and 10 years, which is indicated, in males, by the development of a hooknose. Spawning occurs during the following year, with females depositing about 3,000 eggs per kg in more than one digged and gravel covered nest. One large male usually fertilises the majority of the eggs, after a strong competition (Klemetsen *et al.*, 2003). Although minor variations may occur, influenced by factors such as food availability, temperature and photoperiod, the major periods of the natural breeding cycle of this species are classified as follows (Fig.1): postspawning in late Winter, early vitellogenesis in Spring, late vitellogenesis in Autumn and prespawning in early Winter (Selman *et al.*, 1987; Washburn *et al.*, 1990).

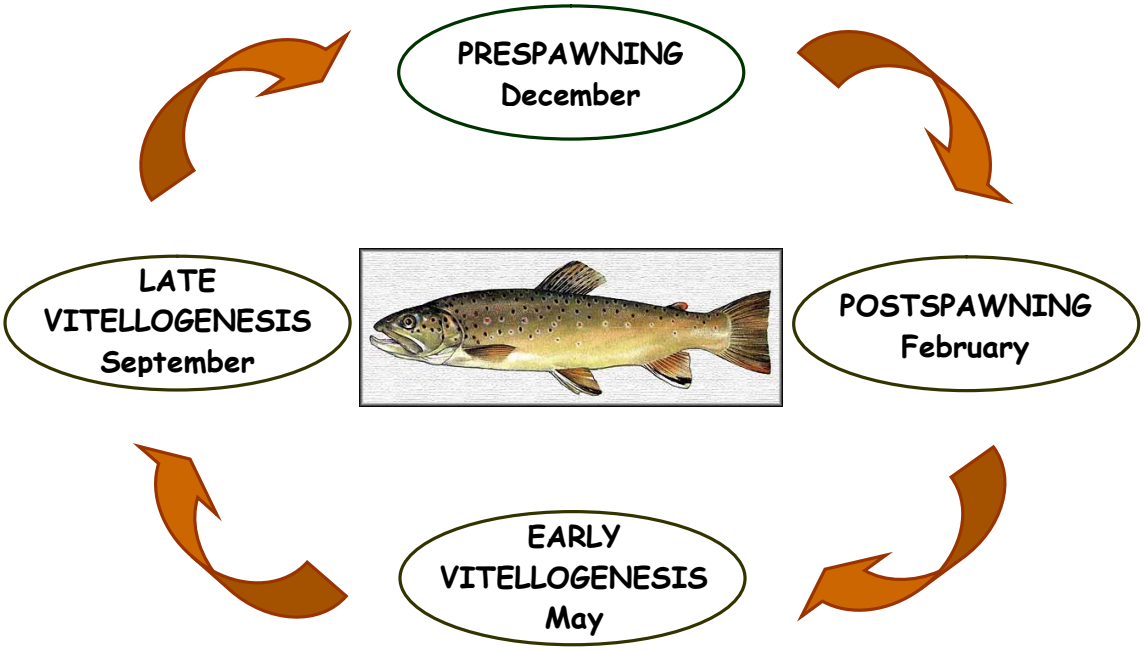


Fig. 1 – The Portuguese brown trout breeding cycle.

As sentinels of environmental pollution disturbances, aquatic species have been gaining attention in recent years, but the current knowledge on fish peroxisomes is yet restricted, which led us to chose a fish as a biological model to our study. Besides its economic interest and the fact of being a native species, the optimum handable size of the specimens, the not-too-long generation time and the availability to acquire good health status fishes, has made brown trout a good option for this study.

1.1.2. Liver metabolism and morphology

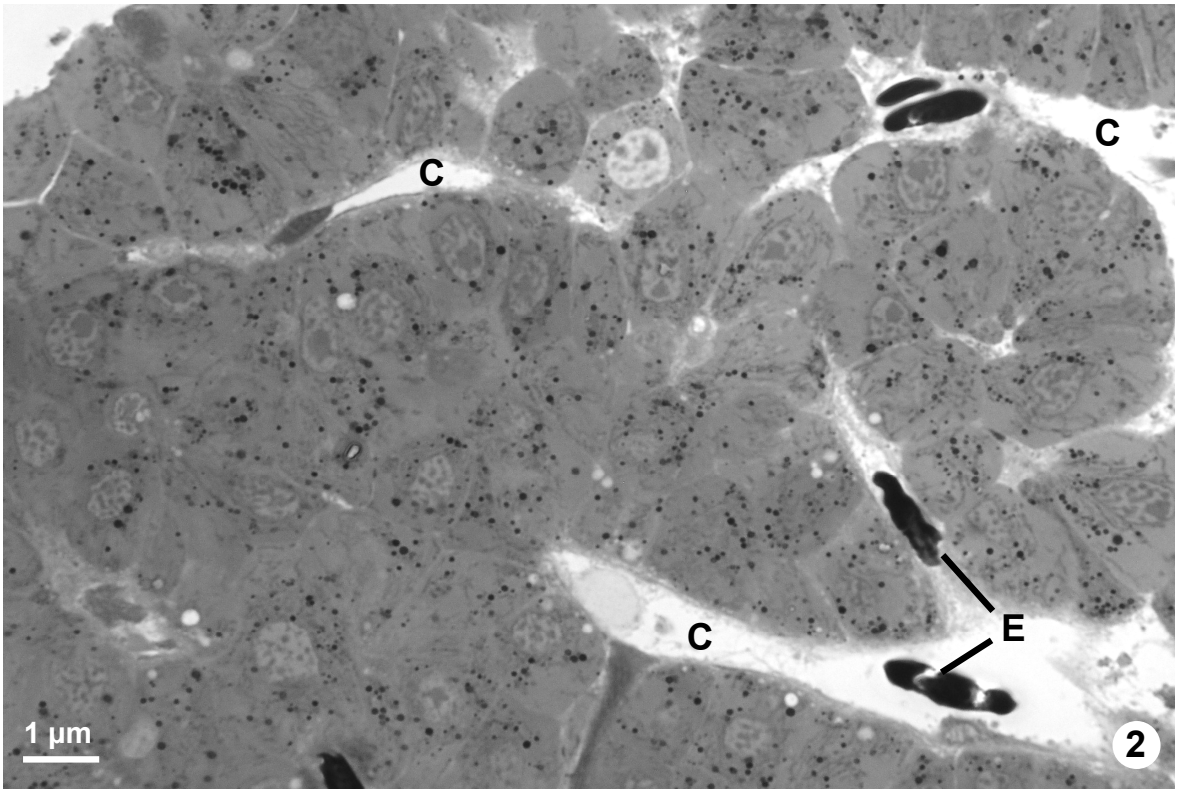
The liver can be considered the major gland of the complex organisms, having a vital role in the integration of diverse physiological and biochemical functions. This organ is involved in the metabolism and excretion of many compounds, including xenobiotics, digestion, accumulation of storage material, and also production of the yolk proteins. In this way, liver developed a peculiar morphology, with interrelating stromal and parenchymal components made of different cell types, all strategically positioned and organized. Since the middle of the late century, fish hepatic histology and cytology have been the subject of

numerous works (Elias and Bengelsdorf, 1952; Hampton *et al.*, 1985; Beresford and Henninger, 1986; Hinton and Couch, 1998) and a crescent focus of interest.

Compared to the traditionally lobulated mammalian liver, fish liver has a somewhat different arrangement, often with no easily distinguishable afferent *versus* efferent veins. Moreover, its structure is also variable from one fish species to another, even within the same family (Reviewed by Rocha and Monteiro, 1999). Over the years, several studies on salmonids liver structure and ultrastructure have been undertaken. Data on the chum salmon, *Oncorhynchus keta*, (Takahashi *et al.*, 1977), coho salmon, *Oncorhynchus kisutch*, (Leatherland, 1982), rainbow trout, *Oncorhynchus mykiss*, (Chapman, 1981; Schär *et al.*, 1985; Hinton and Laurén, 1989), Sevan trout, *Salmo ischchan gegarkuni* Kessel, (Kalashnikova and Kadilov, 1991) and Atlantic salmon, *Salmo salar*, (Robertson and Bradley, 1991; 1992) are available. More recently, a great deal of information on brown trout liver has also been released (Rocha *et al.*, 1994a; 1994b; 1995; 1996; 1997; 1999; 2001a).

In brown trout liver, veins, arterioles and bile ducts can be observed randomly dispersed throughout the parenchyma. Additionally to these isolated elements, a three-dimensional engagement of venous-biliary-arteriolar tracts, venous-biliary tracts, venous-arteriolar tracts and biliary-arteriolar tracts is also present, as shown by serial sectioning analysis. The parenchyma is composed of branched, anastomosing and distorted tubular-like arrangements of the hepatocytes, as seen in Fig. 2, with segments of the biliary system as the axis of the "tubules". A network of sinusoidal capillaries encircles these structural units (Rocha *et al.*, 1995) whose exact three-dimensional structure is still under scrutiny.

Due to its metabolic functions, the hepatocyte is organelle rich and highly organised, showing an apical biliar zone and a basal vascular region (Schramm *et al.*, 1998; Rocha and Monteiro, 1999). The centrally located spherical nucleus is usually surrounded by rough endoplasmic reticulum cisternae, peroxisomes, mitochondria and a few dictyosomes (Fig. 3), all strategically placed in order to assure the maximum functional efficiency. Other organelles, such as lysosomes, are located more at the cell periphery. Some lipid droplets, lipofuscin deposits and large amounts of glycogen granules distributed in clusters throughout the cytosol can also be observed in normal hepatocytes. One or two large nucleoli may be visible in the nucleus, which also presents some heterochromatin spots (Rocha and Monteiro, 1999).



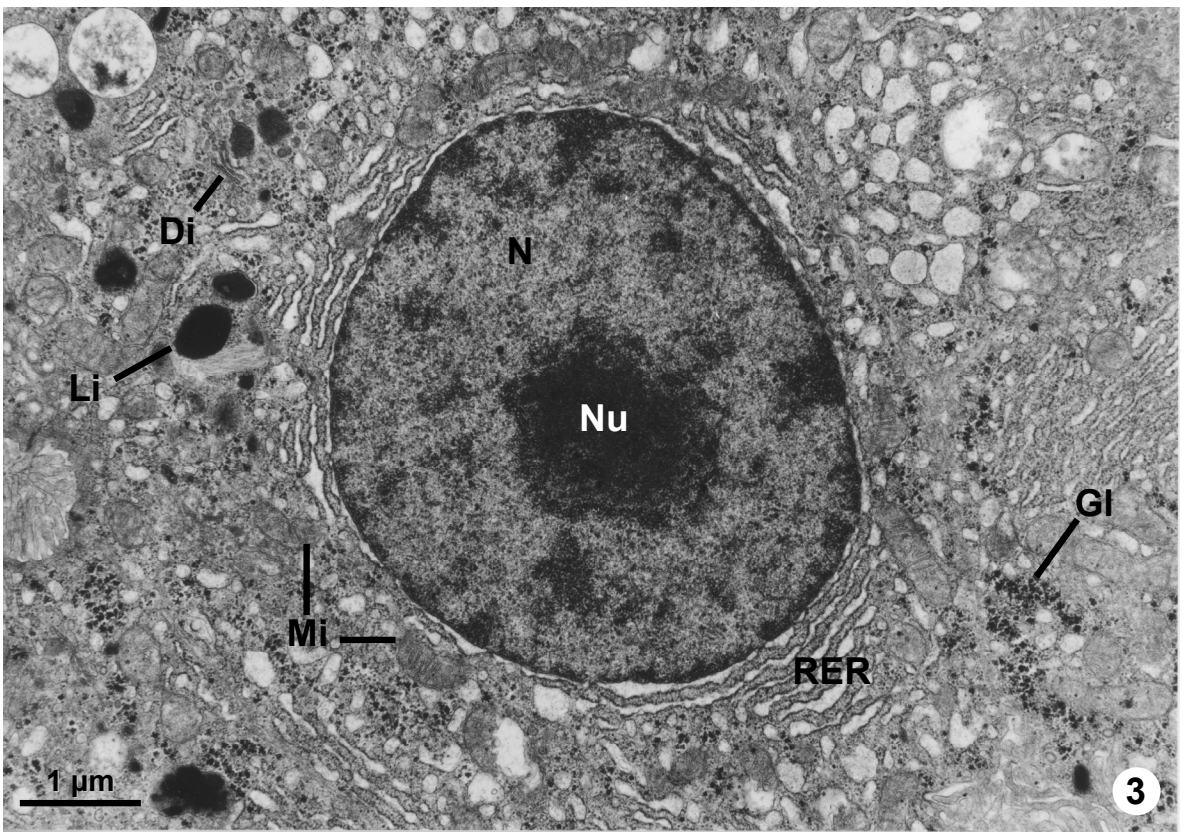


Fig. 2 – Brown trout liver semithin epoxy section, showing hepatocytes often in clear tubular associations, and surrounded by capillaries (C). E – erythrocytes. Fig. 3 – The hepatocytic ultrastructure. N – nucleus, Nu – nucleolus, RER – rough endoplasmic reticulum, Mi – mitochondria, Li – lipids, Di – dictyosome, Gl – glycogen.

Although maintaining its general structure and components, liver is a pretty much dynamic organ, which can undergo morphological transformations to cope with metabolic demands. The relative liver weight of female salmonids varies along the annual breeding cycle, reaching highest values during late vitellogenesis (Takahashi *et al.*, 1977; van Bohemen *et al.*, 1981). Stereological studies on brown trout liver, in both genders and also along the year, were exhaustively made (Rocha *et al.*, 1997; 1999; 2001a). These works focused on liver histological components, on hepatocytes themselves and also on hepatocytic organelles and structures, regarding their relative and absolute numbers, volumes and surfaces.

Because of the importance of peroxisomes in an array of biological processes, including the relationship with the development of hepatic tumours (Reddy and Lalwani, 1983) and the response to environmental water pollutants (Krishnakumar and Casillas, 1995), a special emphasis has been given to the study and quantification of this organelle, not only in the liver of healthy brown trout (Rocha *et al.*, 1999), but also in other fishes living under chemical pollution conditions (Scarano *et al.*, 1994; Oulmi and Braunbeck, 1996; Zahn *et al.*, 1996).

1.2. About peroxisomes

1.2.1. Discovery, morphology and biogenesis

Peroxisomes were discovered and described for the first time in 1954, when Rodhin was observing mouse kidney proximal tubule cells at electron microscope level, during his PhD studies (Rodhin, 1954). Without any clue about their biochemical functions at that time, these new organelles were given the name “microbodies”. More than a decade later, de Duve and Baudhuin adopted the designation “peroxisome” for this hydrogen peroxide producer and catalase containing organelle (de Duve and Baudhuin, 1966). Due to their important metabolic functions, peroxisomes are ubiquitous in eukaryotic cells and have been described in many different kinds of species since then. These include mammals (Hruban and Rechcigl, 1969), birds (Shnitka, 1966), reptiles (Hruban and Rechcigl, 1969), amphibians (Hruban and Rechcigl, 1969), fishes (Veenhuis and Wendelaar Bonga, 1977), molluscs (Lobo-da-Cunha *et al.*, 1994), crustaceans

(Lobo-da-Cunha, 1995), insects (St. Jules *et al.*, 1989), fungi (Maxwell *et al.*, 1975), protozoans (Lobo-da Cunha and Azevedo, 1993) and plants (Huang, 1983), among others. Sometimes a specific designation is used rather than peroxisome. It is the case of the "glyoxisome", found in germinating seeds (Breidenbach and Beevers, 1967; Cooper and Beevers, 1969) and the "glycosome", a related structure present in trypanosomes (Opperdoes and Borst, 1977; Hart and Opperdoes, 1984).

The development of the cytochemical technique based on the peroxidatic reaction of the peroxisomal enzyme catalase using 3,3'-diaminobenzidine (DAB) and hydrogen peroxide as substrate (Novikoff and Goldfischer, 1969) made it possible to unequivocally identify these organelles (Figs. 4 and 7). They are single membrane bounded, usually spherical or oval shaped, with a fine granular matrix, slightly denser than the cytosol (Fig. 4, inset). The peroxisomal approximate diameter – ranging from 0.05 to 3.0 μm – and its number may vary considerably depending on cell type, species, gender, metabolic conditions and stage of development. The rat hepatocytes are among the most peroxisome-rich cells, where they occupy about 1.5% of total cell volume (Beier and Fahimi, 1987).

Crystallized inclusions, named nucleoids or crystalloids, can be easily observed by conventional transmission electron microscopy in the peroxisomes of some species (Figs. 5 - 7). Peroxisome subfractionation (Hruban and Swift, 1964; Lata *et al.*, 1977) and immunocytochemistry (Usuda *et al.*, 1988b) studies on the chemical nature of these crystalloids described them as being urate oxidase, a peroxisomal enzyme, in rat hepatocytes. In agreement with these results, it was verified that some peroxisomes with no nucleoid do not contain urate oxidase. This is the case of human tissues (Goldfischer and Reddy, 1984) and rat kidney (Usuda *et al.*, 1988a; Beard, 1990) peroxisomes. However, in fishes this enzyme has been detected in hepatic peroxisomes with no visible nucleoids, in which it behaves like a peroxisomal matrix soluble enzyme (Noguchi *et al.*, 1979). On the other way, reported nucleoids are not necessarily constituted by urate oxidase. One example of that is the formation of large crystalloids of alcohol oxidase during the exponential growth of the yeast *Hansenula polymorpha* in methanol (Veenhuis *et al.*, 1981). Another peroxisomal enzyme – xanthine oxidase – was also associated with rodent liver crystalloids (Angermüller *et al.*, 1987). Although appearing in peroxisomes of a great number of species, nucleoids are rarely observed in glyoxisomes and glycosomes (Gomez *et al.*, 1974; Rybicka, 1996).

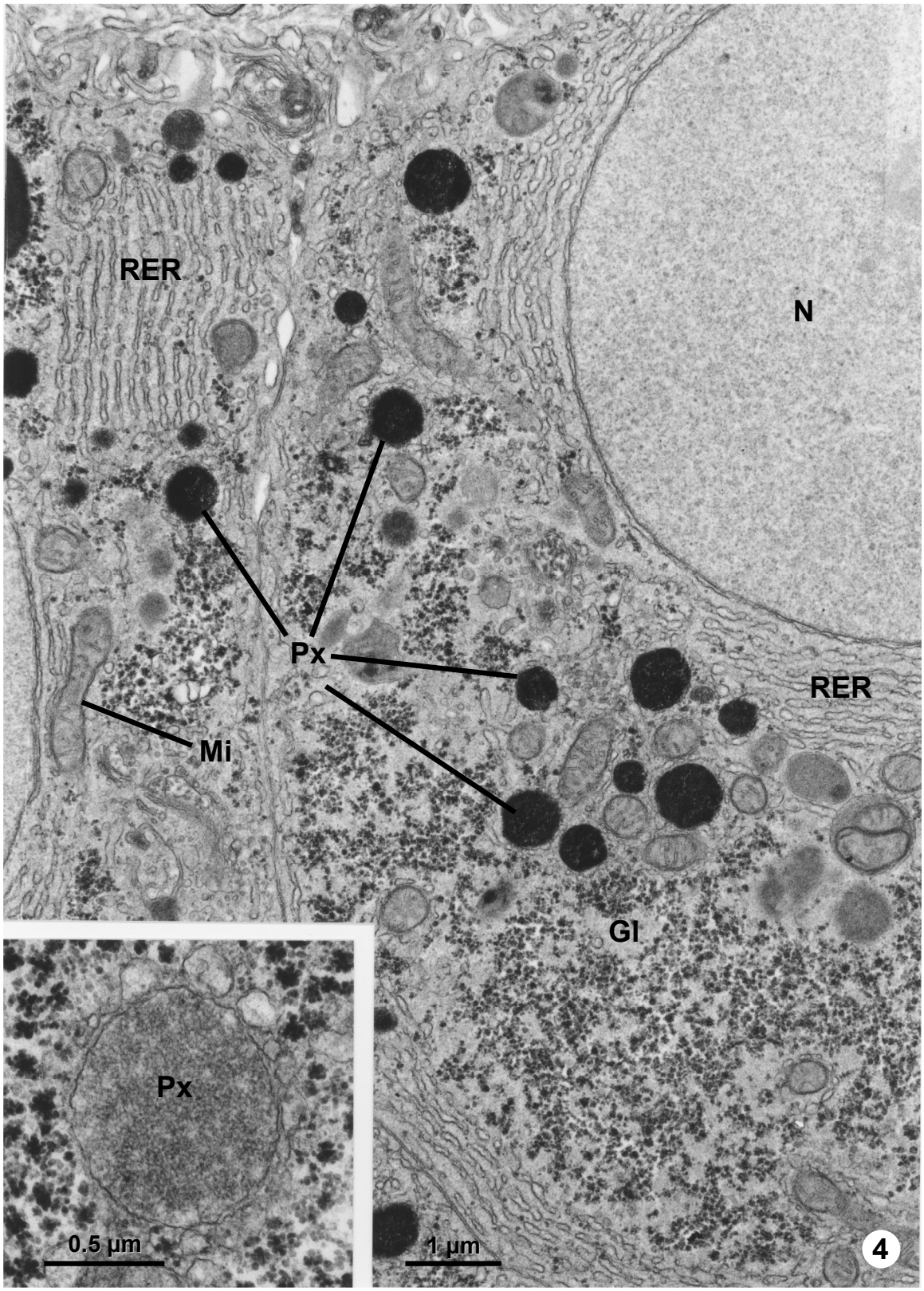
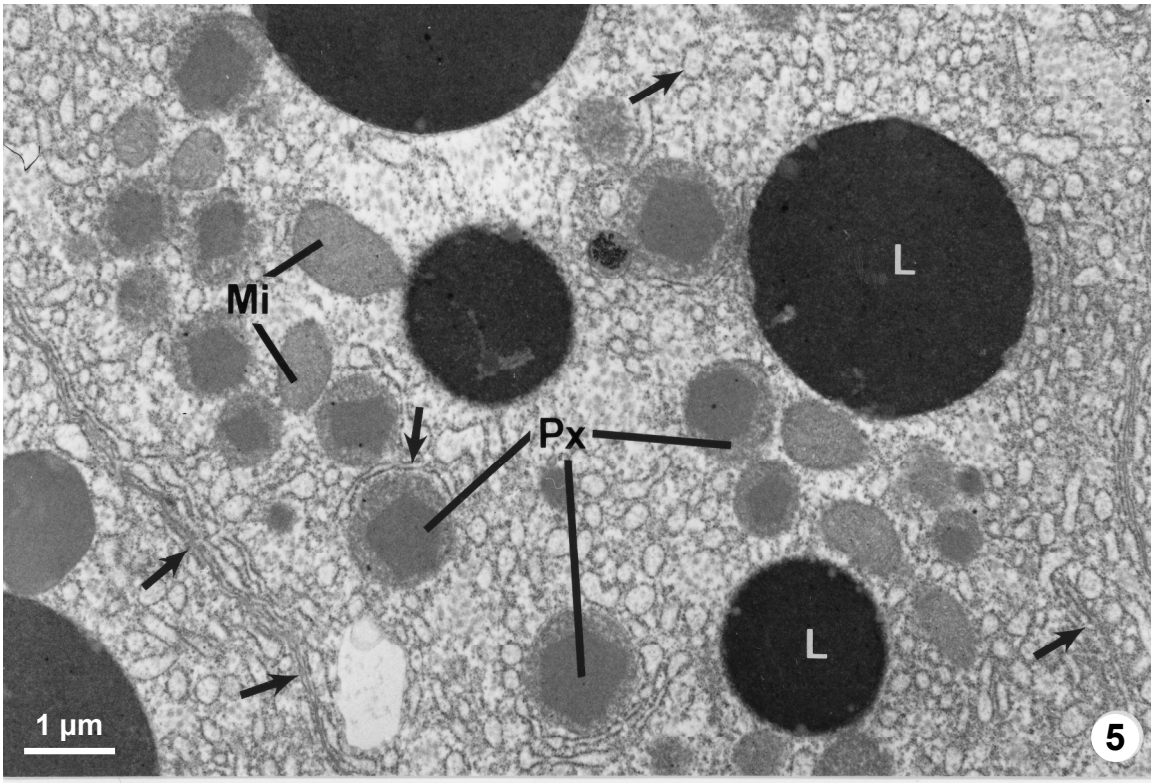
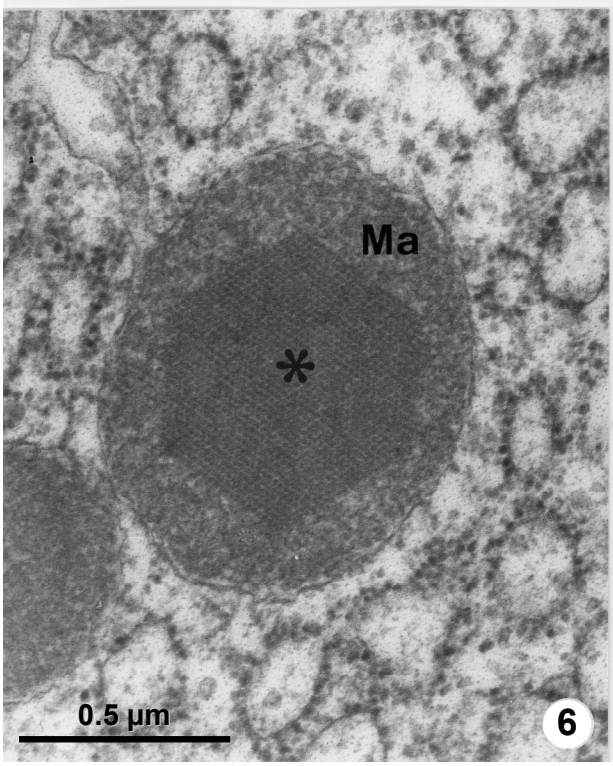
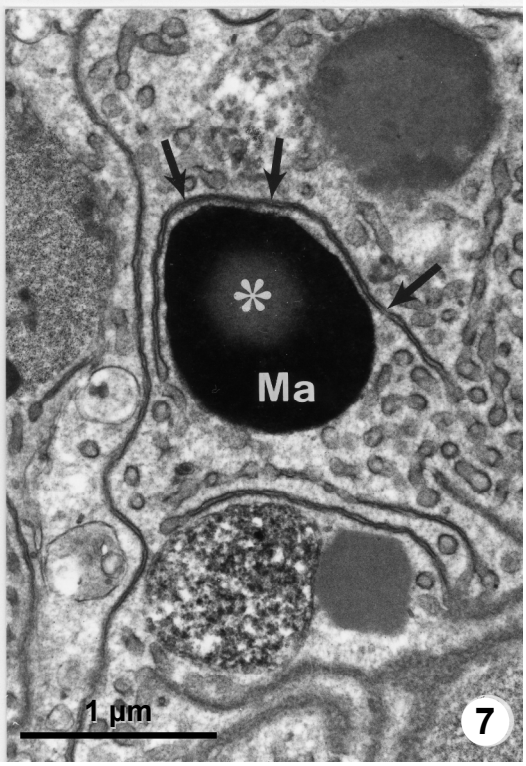


Fig. 4 – Ultrathin section of an *S. trutta* hepatocyte, with peroxisomes (Px) stained after DAB reaction for the detection of catalase, in the proximity of mitochondria (Mi), rough endoplasmic reticulum (RER) and glycogen (Gl). N – nucleus. Inset – Unstained peroxisome (Px) presenting a fine granular matrix with no visible nucleoid.







Figs. 5, 6, 7 – *Gibbula umbilicalis* digestive gland basophilic cells in ultrathin sections. Fig. 5 – Numerous peroxisomes (Px) are seen close to mitochondria (Mi), lipofuscin granules (L) and endoplasmic reticulum cisternae (arrows). Fig. 6 – These peroxisomes show a fine granular matrix (Ma) and a nucleoid (asterisk) with evident hexagonal crystalline structure. Fig. 7 – The product of DAB reaction accumulates in the peroxisomal matrix (Ma) but not in the nucleoid (asterisk). An endoplasmic reticulum cistern (arrows) is located at the peroxisome vicinity.

Another kind of inclusion is sometimes seen strictly associated with the peroxisomal membrane, conferring an angular shape to the organelle (Gorgas and Zaar, 1984; Zaar *et al.*, 1991). These electron dense crystalline formations, known as marginal plates, are usually flat or slightly curved and were identified as L-α-hydroxyacid oxidase B (Zaar *et al.*, 1991; Yokota and Hashimoto, 1995). Some peroxisomes, like the rhesus monkey renal peroxisomes (Tisher *et al.*, 1968), present two marginal plates, appearing elongated and narrow. In the kidney cortex of beef and sheep (Zaar and Fahimi, 1991), the existence of multiple marginal plates was reported. Although mainly observed in renal cells, these structures are also seen in other cell types, such as glial tumour and sebaceous gland cells (Sima, 1980; Gorgas and Zaar, 1984).

Peroxisomes have frequently been observed in strict association with the endoplasmic reticulum. In 1964, Novikoff and Shin reported the existence of structural continuities between both organelles in rat liver, a feature that was later described in several tissues and species (Novikoff and Novikoff, 1972; Reddy and Svoboda, 1973; Zaar and Gorgas, 1985). Ever since these observations were reported, an attempt has been made by many investigators to establish if the peroxisomes originate from the endoplasmic reticulum. Indeed, morphological analysis of numerous electron microscopy pictures in several species suggested an endoplasmic origin of peroxisomes, a theory mainly defended by the above mentioned investigators, even after reports of cytochemical and biochemical studies pointing towards an opposite idea. Fahimi demonstrated, in a first approach, that rat hepatocytes endoplasmic reticulum lumen was negative to catalase activity (Fahimi, 1969). Subsequent studies based on serial sectioning analysis and cytochemical reactions indicated that peroxisomes and endoplasmic reticulum were distinct organelles, with catalase localized inside the former and peroxidase inside the latter (Fahimi *et al.*, 1976). This result was later corroborated by the cytochemical localization of glucose-6-phosphatase activity in the endoplasmic reticulum and not in membrane structures associated with peroxisomes (Shio and Lazarow, 1981). Biochemical techniques reinforced this theory by proving that catalase and urate oxidase are synthesised in free ribosomes (Goldman and Blobel, 1978) and that catalase is translocated to peroxisomes without N-terminal cleavage (Robbi and Lazarow, 1982). Posterior studies led to the same conclusions for the remaining peroxisomal proteins, either matrix or membrane proteins (Ozasa *et al.*, 1983; Fujiki *et al.*, 1984; Miura *et al.*, 1984; Rachubinski *et al.*, 1984; Lazarow and Fujiki, 1985; Fujiki *et al.*,

1986; Köster *et al*., 1986; Suzuki *et al*., 1987; Imanaka *et al*., 1996). This theory is also supported by numerous ultrastructural studies in rat, fungi, yeast and crab, in which peroxisome matrix density maturation, peroxisome membrane evaginations and peroxisome division were observed (Tsukada *et al*., 1968; Legg and Wood, 1970; Osumi and Fukuzumi, 1975; Fahimi *et al*., 1993a; 1993b; Lobo-da-Cunha, 1995). According to a model established for rat hepatocytes, peroxisomes form membrane loops (which were previously mistaken with endoplasmic reticulum extensions) capable of incorporating peroxisomal membrane and matrix proteins. These new compartments finally separate, continuing to grow afterwards (Fahimi *et al*., 1993a; 1993b). Peroxisomal membrane loops were also observed in brown trout hepatocytes, clearly connected with the organelle (Rocha *et al*., 1999). In this way, the theory that peroxisomes develop from fission of pre-existing ones, subsequently importing the necessary peroxisomal proteins, and implying at least one original peroxisome in each new cell after mitosis, has gained acceptation.

New controversy about this issue was shed after recent reports in both yeast and mammals of the appearance of new peroxisomes in cells which lacked these organelles (Matsuzono *et al*., 1999; South and Gould, 1999; Sacksteder and Gould, 2000) and the finding that some yeast peroxisomal membrane proteins are only found in endoplasmic reticulum and peroxisomes (Titorenko *et al*., 1997; Titorenko and Rachubinski, 2001; Faber *et al*., 2002). Hence, one of the current believes is in the direction of a semi-autonomous origin of peroxisomes, with a contribution of the endoplasmic reticulum on their membrane formation (Geuze *et al*., 2003). In this line, a rather revolutionary concept about peroxisome biogenesis was recently proposed, in which a group of peroxins enters the endoplasmic reticulum, concentrates in special regions and finally captures a piece of its membrane, banishing the resident proteins. The new compartment is then detached to form pre-peroxisomes that undergo maturation (Tabak *et al*., 2006). Despite being a conciliatory answer to an ancient question, this theory raises new ones. For instance, the mechanisms by which peroxins are directioned to the endoplasmic reticulum or how small vesicles are released from it.

Although peroxisomes often appear approximately round in isolated sections, there is evidence for the existence of a peroxisomal reticulum, at least in some occasions. After the studies of Lazarow and coworkers (1980), suggesting that peroxisomes may be interconnected, serial section reconstruction works proved the existence of such a network (Gorgas, 1985; Yamamoto and Fahimi, 1987).

Later on, this peroxisomal reticulum was also observed *in vivo* in cultured cells by fluorescent microscopy and shown to have a dynamic behaviour, with peroxisomes transiently linked to each other (Schrader *et al.*, 2000).

1.2.2. Metabolic functions

Since the first time an inherited disease was associated to a peroxisomal dysfunction (Goldfischer *et al.*, 1973), peroxisomes received a great deal of attention in terms of metabolic studies. In mammals, more than 60 peroxisomal enzymes involved in important metabolic pathways (Fig. 8) were already identified (Reviewed by Singh, 1997).

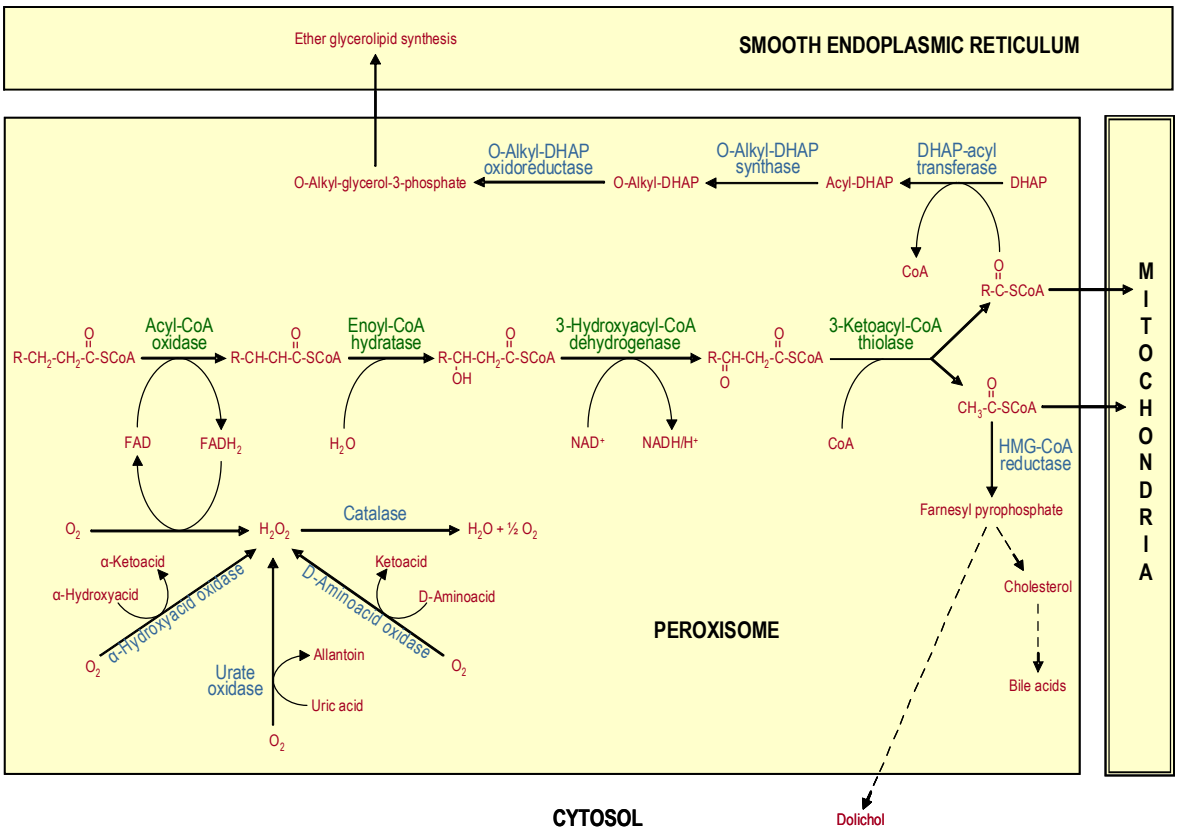


Fig. 8 – Major metabolic pathways occurring in mammalian hepatic peroxisomes. The β-oxidation enzymes are shown in green. Shortened fatty acids can be further oxidized in mitochondria or used as substrates for the biosynthesis of ether glycerolipids in the smooth endoplasmic reticulum. Acetyl-CoA can enter the mevalonate pathway and give rise to dolichol, cholesterol and bile acids. Adapted from Fahimi *et al.*, 1993a.

In animal cells, **β-oxidation** of fatty acids occurs in peroxisomes, as well as in mitochondria (Mannaerts and Van Velhoven, 1993). However, despite the similarity of the mechanisms in these organelles, there are significant differences between the two systems, mainly concerning the intervenient enzymes and the nature of substrates. It is now clearly established that mitochondria are able to β-oxidize medium and long chain fatty acids but the initial steps of the β-oxidation of very long chain fatty acids and polyunsaturated fatty acids take place preferentially in peroxisomes (Kondrup and Lazarow, 1985; Hiltunen *et al.*, 1986; Chance and McIntosch, 1994). It was also demonstrated that peroxisomes do not act only as fatty acids chain-shorteners, but they also carry out the whole β-oxidation pathway of very long chain fatty acids ($>C_{22}$) in certain cell types like hepatocytes, brain cells and skin cultured fibroblasts (Chance and McIntosch, 1994; Reddy and Mannaerts, 1994; Singh, 1997).

Fatty acids can only be β-oxidized in the form of their acyl-CoA derivatives. This activation step is performed by different acyl-CoA synthetases, located in the peroxisomal membrane (Singh, 1992). Once activated, the acyl-CoA esters enter the β-oxidation pathway to follow a sequence of four reactions by three different enzymes (Fig. 8). Acyl-CoA oxidases dehydrogenate acyl-CoA esters to 2-trans-enoyl-CoA. These are hydrated to L-3-hydroxiacyl-CoA and then dehydrogenated to 3-keto-acyl-Coa by the multifunctional enzymes acting both as enoyl-CoA hydratases and as L-3-hydroxiacyl-CoA dehydrogenases. Finally, 3-keto-acyl-CoA thiolases cut these molecules to free acetyl-CoA and an acyl-CoA derivative two carbon atoms shorter than the original, which re-enters the β-oxidation chain (Lazarow, 1978; Hashimoto, 1987; Reddy and Mannaerts, 1994; Singh, 1997).

In parallel with the β-oxidation of fatty acids, the chain-shortening of certain bile acids intermediates occurs through a β-oxidation process with a distinct set of enzymes located exclusively in peroxisomes (Kase *et al.*, 1986; Björkhem, 1992; Russel and Setchell, 1992). Moreover, it was recently shown that the entire β-oxidation of 2-methyl branched-chain fatty acids, such as di- and trihydroxycholestanoic acid and pristanoic acid, is an exclusive peroxisomal task (Ferdinandusse *et al.*, 2001).

Peroxisomal β-oxidation is also essential for the metabolization of many other chemicals, such as medium and long chain dicarboxilic acids (Kølvraa and

Gregersen, 1986; Ferdinandusse *et al.*, 2004), prostaglandins (Schepers *et al.*, 1988) and xenobiotics (Yamada *et al.*, 1987; Yoshida *et al.*, 1990).

Concerning the final metabolites and the controversial localization of some enzymes, **purine catabolism** pathway (Fig. 9) is rather variable among species. Nonetheless, some steps are known to take place in peroxisomes.

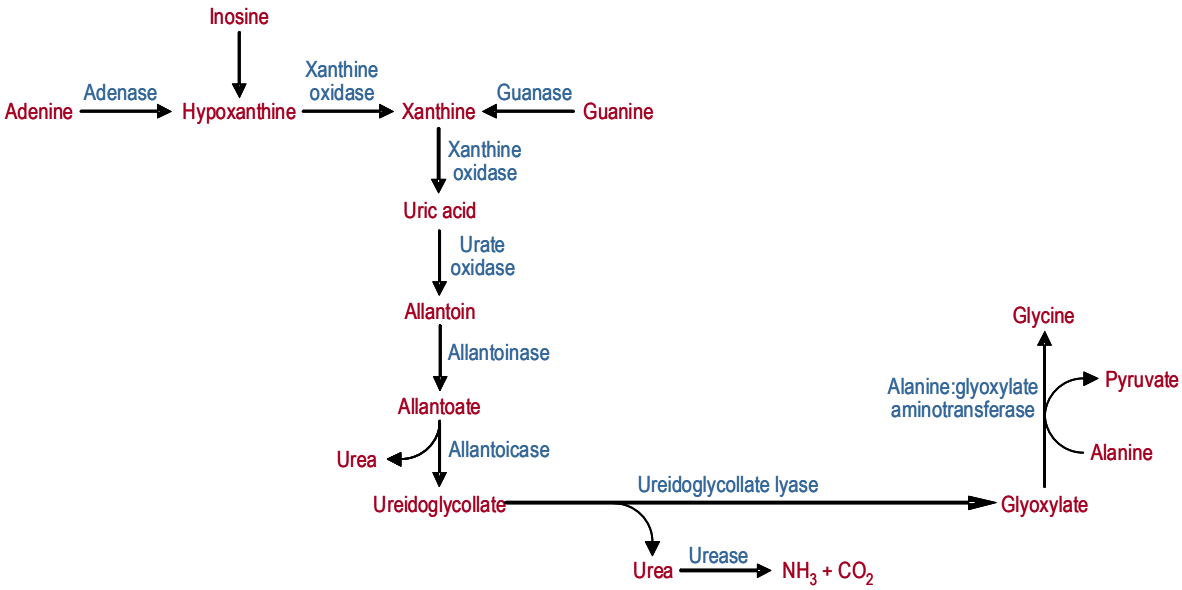


Fig. 9 – Route for the degradation of purines and reutilization of purine carbon skeletons in the liver. Adapted from Sakuraba *et al.*, 1996.

Degradation of purines to uric acid is generally carried out in the cytosol, with few exceptions: xanthine oxidase was reported in hepatic and renal peroxisomes of birds and amphibians (Scott *et al.*, 1969) and rat hepatic peroxisomes (Angermüller *et al.*, 1987). During the evolutionary process, humans and some hominoid primates have lost urate oxidase due to mutations in the corresponding gene (Wu *et al.*, 1992). In this way, uric acid is the final product of purine degradation in these primates, as well as in birds, terrestrial reptiles, some insects and certain gastropods. Other mammals and several reptiles are able to catabolise uric acid to allantoin through a reaction catalyzed by urate oxidase (Friedman *et al.*, 1985; Usuda *et al.*, 1988a). This enzyme has been largely described in the peroxisome nucleoids of many species (Lata *et al.*, 1977; Usuda *et al.*, 1988b; Usuda *et al.*, 1994) and even in the peroxisomal matrix of some fishes and crustaceans (Noguchi *et al.*, 1979). Some teleost fishes and

amphibians further degrade allantoin to allantoic acid through allantoinase, and allantoic acid to urea and glyoxylate by reactions catalyzed by allantoicase and ureidoglycollate lyase, respectively (Scott *et al.*, 1969). Although the intracellular localization of allantoinase and allantoicase in some marine fishes and crustaceans is attributed to peroxisomes (Noguchi *et al.*, 1979; Hayashi *et al.*, 1989b), it has also been reported to be respectively mitochondrial and cytosolic by immunocytochemical studies in frog liver and kidney (Yeldandy *et al.*, 1995) and, in some freshwater fishes, allantoinase is only found in cytosol (Fujiwara *et al.*, 1989). In marine invertebrates and crustaceans, urea is finally degraded to NH_3 and CO_2 through the action of urease (Hayashi *et al.*, 2000).

Peroxisomal enzymes are also implicated in the **synthesis of cholesterol, dolichol** and other important isoprenoids playing a crucial role in the cellular homeostasis. Cholesterol is widely known as an essential constituent of cell membranes, being a major determinant of membrane fluidity. It is also a precursor of bile acids and steroid hormones (Bloch *et al.*, 1943; Payne and Hales, 2004). Although ubiquitarily present in plant and animal tissues, dolichol has a more intriguing biological role. So far, it was only proven to be involved in protein glycosilation (Leloir, 1977; Burda and Aebi, 1999) and suggested to behave as a free radical scavenger (Bizzarri *et al.*, 2003).

Cholesterol and dolichol syntheses follow the same route until the formation of farnesyl pyrophosphate from acetyl-CoA through the mevalonate pathway (Goldstein and Brown, 1990) and then diverge (Fig. 10). This pathway is shared by cytosol, peroxisomes, endoplasmic reticulum and mitochondria (Mannaerts and Van Velhoven, 1993; Aboushadi *et al.*, 1999; Olivier *et al.*, 2000). Peroxisomes contain all the enzymes necessary for the conversion of acetyl-CoA to farnesyl pyrophosphate but the first two steps are duplicated in the cytosol and mitochondria, and the third one also occurs in the endoplasmic reticulum. The rest of the pathway is thought to be exclusively peroxisomal (Olivier *et al.*, 2000). For cholesterol synthesis, the intervention of the endoplasmic reticulum is obligatory at this point, but the process can be completed again in peroxisomes, as well as in the endoplasmic reticulum (Fig. 10). This duplication suggests the existence of different destinations for cholesterol, depending on where it comes from. It is thought that the endoplasmic reticulum contributes for cholesterol used in lipoprotein and membrane generation, whereas peroxisomes originate

cholesterol implicated in bile acids synthesis and transport (Singh, 1997). For dolichol and other isoprenoids synthesis, farnesyl pyrophosphate is transferred to the cytosol and further transformed (Aboushadi *et al.*, 1999).

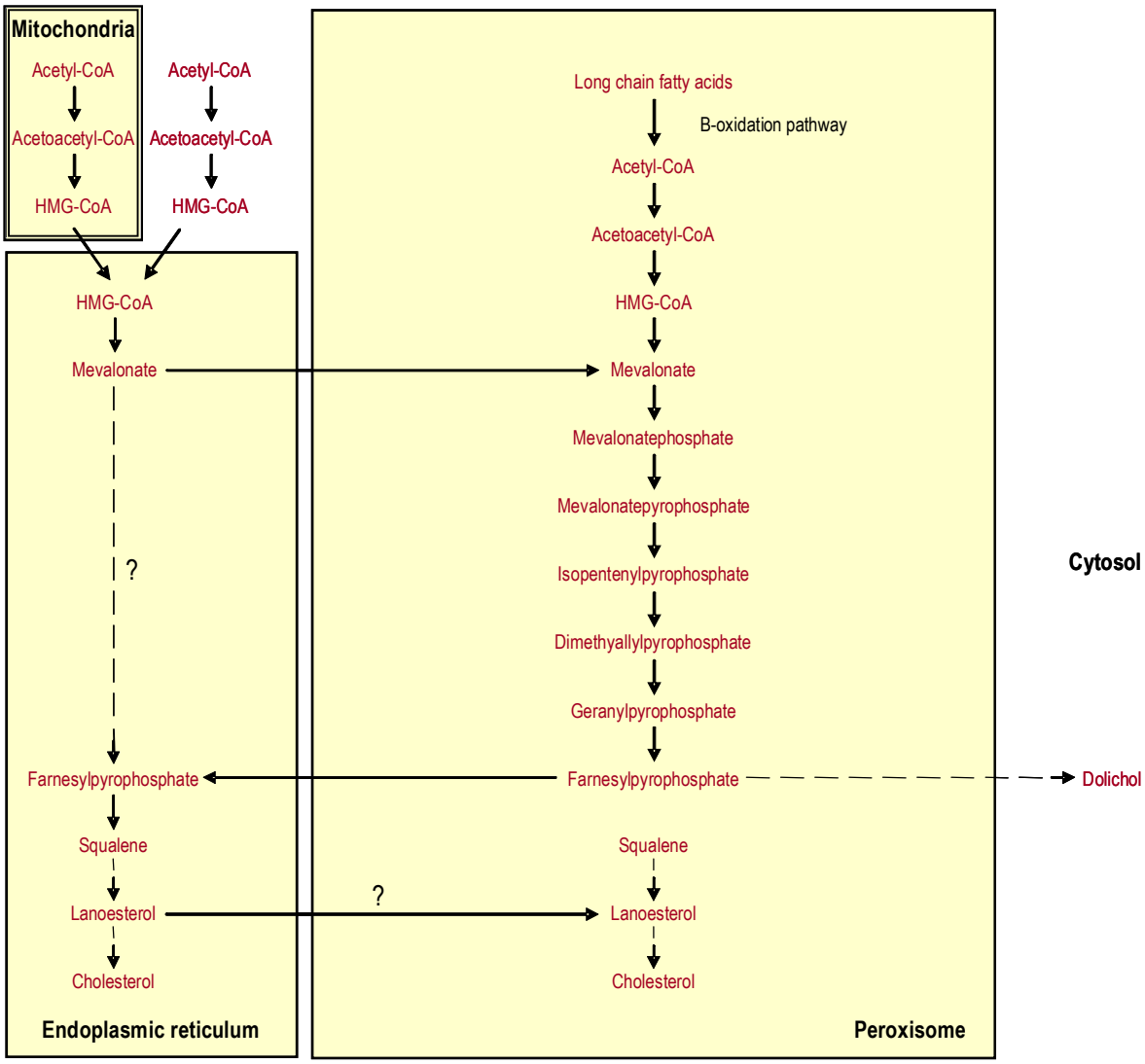


Fig. 10 – Current model of the subcellular compartmentalization of cholesterol biosynthesis. Conversion of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA occurs in the cytosol, peroxisomes, and mitochondria. The further conversion of 3-hydroxy-3-methylglutaryl-CoA to mevalonate occurs both in endoplasmic reticulum and in peroxisomes. However, the conversion of mevalonate to farnesyl pyrophosphate occurs predominantly in the peroxisomes. The further metabolism of farnesyl pyrophosphate to squalene proceeds exclusively in the endoplasmic reticulum, and the final conversion of lanosterol to cholesterol occurs in the endoplasmic reticulum and may also be localized in peroxisomes. Adapted from Olivier *et al.*, 2000.

Degradation of cholesterol to **produce bile acids** occurs in liver following two possible pathways, depending on species and enzyme availability (Chiang, 1998). The classic pathway requires the intervention of a set of enzymes located in cytosol, endoplasmic reticulum, mitochondria and peroxisomes (Pederson, 1993; Chiang, 1998). Peroxisomes are responsible for the β-oxidation reactions that shorten the cholesterol ring side chain of the bile acids intermediates 3α,7α-dihydroxy-5β-cholestanoic acid and 3α,7α,12α-trihydroxy-5β-cholestanoic acid to produce chenodeoxycholic acid and cholic acid, respectively (Kase *et al.*, 1986; Björkhem, 1992; Russel and Setchell, 1992; Pederson, 1993).

Among the peroxisomal pool of enzymes, stands a group of enzymes involved in **amino acids catabolism**. D-Amino acid oxidase, responsible for the oxidation of the neutral and basic D-isomers, producing the corresponding ketoacids, ammonia and hydrogen peroxide, was the first to be discovered (de Duve and Baudhuin, 1966). An enzyme that oxidizes the acidic D-isomers, D-aspartate oxidase, was also found in peroxisomes (Zaar *et al.*, 1989), as well as L-α-hydroxyacid oxidases A and B (Angermüller *et al.*, 1986), alanine:glyoxylate aminotransferase (Noguchi, 1987) and L-pipecolate oxidase (Wanders *et al.*, 1989). Since D-amino acids have no known metabolic meaning in mammals, the physiological roles of D-amino acid oxidase and D-aspartate oxidase remain unclear. Nevertheless, they are present in the peptidoglycans of bacterial cell walls and some results suggest that these enzymes might have a possible intervention in the catabolism of D-amino acids from the microbial intestinal flora (Hoeprich, 1965; Konno *et al.*, 1989). More recently, certain D-amino acids have been found in nervous tissue from molluscs, amphibians and vertebrates, either in the free form or as residues of certain neuropeptides, where they seem to participate in the modulation of important neuronal functions (Fujisawa *et al.*, 1992; Hashimoto *et al.*, 1993; Yasuda-Kamatini *et al.*, 1995; Zaar *et al.*, 2002). D-aspartate oxidase was suggested to be involved in the degradation of D-isomers of acidic dicarboxilic amino acids, which can behave as nonphysiological neurotransmitter ligands, and cause disturbances in the central nervous system neuronal function (Zaar, 1996). Another hypothesis was raised, suggesting that these oxidases use substrates other than D-amino acids, such as cysteamin, L-cysteine or L-cysteinylglycine, originating products possibly integrated in intracellular messenger systems for several hormones, like insulin (Hamilton *et al.*, 1987).

L-α-Hydroxyacid oxidase A, located in hepatic and sometimes renal peroxisomes (Yokota *et al.*, 1985), is also known as glycolate oxidase and oxidizes preferably short chain aliphatic L-α-hydroxyacids to produce the corresponding α-ketoacids and hydrogen peroxide (Mannaerts and Van Veldhoven, 1993). Forming the marginal plates of renal peroxisomes, L-α-hydroxyacid oxidase B acts on long chain aliphatic L-α-hydroxyacids, aromatic L-α-hydroxyacids and L-amino acids, generating the same products as the A isotype and also ammonia, in the case of amino acids (Zaar *et al.*, 1991; Mannaerts and Van Veldhoven, 1993; Masters and Crane, 1995). Besides catalyzing the oxidation of glycolate to glyoxylate, the A isotype oxidizes glyoxylate to oxalate, as well (Yanagawa *et al.*, 1990; Mannaerts and Van Veldhoven, 1993).

In mammals, alanine:glyoxylate aminotransferase can be found in peroxisomes and/or in mitochondria, depending on the species (Noguchi, 1987; Noguchi and Fujiwara, 1988). In general, this enzyme presents both alanine:glyoxylate aminotransferase and serine:pyruvate aminotransferase activities, but in rodent liver it recognizes a wide range of amino acids as amino-donors (Noguchi, 1987; Noguchi and Fujiwara, 1988; Danpure, 1993). A deficiency in this enzyme results in the accumulation of glyoxylate and subsequent excessive oxalate production (Yanagawa *et al.*, 1990), which leads to calcium oxalate deposition and renal failure, a pathology known as primary hyperoxaluria type I in humans (Danpure and Jennings, 1986).

Degradation of L-Lysine in α-aminoadipate follows two possible ways: the saccharopine pathway and the L-pipecolate pathway (Mannaerts and Van Veldhoven, 1993). The former seems to occur in virtually all tissues except for the brain, where the latter predominates (Hutzler and Dancis, 1968; Chang, 1982). L-Pipecolate oxidase, which transforms L-pipecolate in Δ^1-piperideine-6-carboxylate, was identified so far in peroxisomes of man and rhesus monkey (Wanders *et al.*, 1989; Mihalik *et al.*, 1991) and in mitochondria of other mammals (Mihalik and Rhead, 1991). L-Pipecolate accumulates in tissues and fluids of patients with peroxisomal deficiency (Lazarow and Moser, 1989).

The carbon skeletons resulting from these oxidative transaminations and deaminations can be employed in gluconeogenesis, a glucose synthesizing process occurring in liver and kidney cortex of mammals (Mannaerts and Van Veldhoven, 1993).

Polyamine oxidase plays a key role in **polyamines metabolism**, catabolizing spermine, N-acetylspermine, spermidine and N-acetylspermidine, molecules involved in the regulation of cell growth and differentiation (Hölttä, 1977; McCann *et al*., 1987). The activity of polyamine oxidase was shown to increase under conditions of cell and peroxisome proliferation such as administration of clofibrate, a peroxisome proliferator which also causes hepatomegaly (Hayashi *et al*., 1989a), but its intracellular localization is controversial: it has been referred to be both cytosolic and peroxisomal (Hölttä, 1977; Libby and Porter, 1987; Tsukada *et al*., 1988), though a more recent ultrastructural study in rat tissues demonstrated that polyamine oxidase activity in kidney and liver is exclusively attributed to peroxisomes and in the duodenum to microperoxisomes (van den Munckhof *et al*., 1995).

Additionally to the cytosol, mitochondria and endoplasmic reticulum, also peroxisomes are implicated in **respiration and oxygen metabolism** (de Duve and Baudhuin, 1966; Singh, 1997).

Along with with substrates oxidation, oxygen is consumed to generate hydrogen peroxide by specific oxidoreductases (Fig. 8). Several types of substrates can be used as electron donors in these reactions. The oxidases involved in amino acids catabolism, polyamine oxidase, urate oxidase, nitric oxide synthase and the acyl-CoA oxidases palmitoyl-, pristanoyl- and trihydroxycoprostanoyl-CoA oxidases were already identified in mammalian peroxisomes (Reviewed by Schrader and Fahimi, 2004). In addition to the non-radical reactive oxygen species (ROS) hydrogen peroxide (H_2O_2), these organelles also generate trace amounts of superoxide anion ($O_2\cdot^-$) radicals: the proteolytic conversion, under certain conditions, of xanthine dehydrogenase to xanthine oxidase in the peroxisomal matrix (Engerson *et al*., 1987) and several cytochrome-like peroxisomal membrane proteins recently identified in plant peroxisomes (López-Huertas *et al*., 1997; 1999) constitute sources of $O_2\cdot^-$, which originates the extremely reactive hydroxyl (OH·) in the presence of transition metals (Lazarow *et al*., 1980).

Decomposition of H_2O_2 is made by catalase, the most abundant peroxisomal enzyme (de Duve and Baudhuin, 1966), through two possible ways, depending on the conditions: the catalytic reaction, which involves two H_2O_2 molecules and the peroxidatic reaction, in which one H_2O_2 molecule reacts with another substrate, such as ethanol, methanol, phenols or nitrites (Oshino *et al*., 1973). Other

antioxidant enzymes participating in the detoxification of ROS are glutathione peroxidase and peroxiredoxin I for the metabolization of H_2O_2, and Mn-superoxide dismutase and Cu,Zn-superoxide dismutase for detoxification of $O_2 \cdot^-$ (Schrader and Fahimi, 2004). Regardless of their cytosolic, nuclear, mitochondrial or lysosomal existence, all these enzymes have been also found in peroxisomal matrix or membrane (Orbea *et al.*, 2000; Schrader and Fahimi, 2004).

Peroxisomes are also involved, in one way or another, in several other processes, such as glycerolipidic ethers biosynthesis, hexose monophosphate production, phytanic acid α-oxidation and pipecolic acid metabolism (Mannaerts and Van Veldhoven, 1993; Singh, 1997).

1.2.3. Regulation by estrogens

The presence of peroxisomes in mammal steroidogenic tissues, such as adrenal cortex, ovary lutein cells and testis Leydig cells (Magalhães and Magalhães, 1971; Hruban *et al.*, 1972; Reddy and Svoboda, 1972) denotes the importance of these organelles in steroid synthesis. In fact, they are involved in the biosynthetic pathway of cholesterol, the precursor of steroid hormones, as previously referred. Moreover, peroxisomes also play a role in the regulation of steroid levels, by containing 17β-hydroxyesteroid dehydrogenase type IV, a key enzyme in the oxidation of estradiol to estrone (Markus *et al.*, 1995).

On the other hand, peroxisomes themselves can be regulated by estrogenic compounds. Peroxisome proliferation was observed in the mallard ducks uropygial glands upon estradiol treatment, as well as the induction of the peroxisomal enzymes involved in the formation of female pheromones (Bohnet *et al.*, 1991). A stereological approach through transmission electron microscopy used to investigate the relationship between gonad development and hepatic peroxisomes in brown trout demonstrated that a significant reduction in peroxisome individual and total volume per cell occurs in late vitellogenic females, when estradiol reaches highest levels (Rocha *et al.*, 1999). This study was later complemented with a biochemical component, which revealed a simultaneous decrease of some peroxisomal enzymes activities, especially of urate oxidase, which almost disappeared (Rocha *et al.*, 2001b; 2004; Resende *et*

al., 2005). A recent study on zebrafish liver peroxisomes (Ortiz-Zarragoitia and Cajaraville, 2005) revealed that the decrease in peroxisome individual volume caused by estrogenic compounds in induction experiments can be accompanied by an increase in their number, which results in an overall increase in peroxisome total volume. The activity of the β-oxidation enzyme acyl-CoA oxidase was also enhanced in the same conditions. Disregarding differences in the methodology used, such as estrogen dosages, it is possible that species differences in sensitivity, liver physiology and regulatory mechanisms can lead to somewhat divergent results. Anyhow, it is evident that a relationship between estradiol and peroxisomes do exist.

1.2.4. Fish peroxisomes

Although several fish organs such as intestine and kidney (Connock, 1973; Veenhuis and Wendelaar Bonga, 1977; Resende *et al*., 2005) have been the subjects for morphologic and functional peroxisomal studies, the majority of the data available on fish peroxisomes concerns to the liver.

Depending on the tissues, species, gender, developmental stage and external conditions that influence the metabolic state, peroxisomes can vary substantially and fish peroxisomes are no exception. Its diameters can be as small as 0.2 μm in the eel, *Anguilla anguilla* L., (Braunbeck and Völkl, 1991) or as large as 3 μm in gray mullet, *Mugil cephalus*, (Orbea *et al*., 1999). Curiously, it was observed in the golden ide, *Leuciscus idus melanotus*, liver that cold adaptation induces the emergence of a group of smaller peroxisomes and an increase in catalase activity (Braunbeck *et al*., 1987). Hepatic fish peroxisomes size, number and enzymatic activity are also variable with gender and season, as referred above (Rocha *et al*., 1999; 2001b; 2004; Resende *et al*., 2005). A common characteristic that seems to be shared by almost all the species is the absence of nucleoid in hepatic fish peroxisomes, although a noncrystalline dense core was very rarely observed in the carp, *Cyprinus carpio*, (Kramar *et al*., 1974).

Fish peroxisomes are somehow adapted to the specific metabolic demands of aquatic vertebrates. Of special relevance is the fact that these animals rather use lipids than carbohydrates as their main source of energy (Cowey and Sargent, 1977; Watanabe, 1982). In this way, the metabolism of fatty acids is one of the

most important functions of fish peroxisomes. The β-oxidation enzymes acyl-CoA oxidase and multifunctional enzyme were detected in the peroxisomes of rainbow trout, *Oncorhynchus mykiss*, (Yang *et al.*, 1990), in the channel catfish, *Ictalarus punctatus*, (Gallagher and Di Giulio, 1991), in the Japanese medaka, *Oryzias latipes*, (Scarano *et al.*, 1994) and in the gilthead sea bream, *Sparus aurata*, (Pedrajas *et al.*, 1996). After the peroxisomal chain-shortening steps, fatty acids are translocated to mitochondria to proceed with the β-oxidation process. Carnitine acetyltransferase, the enzyme that transports acyl groups across mitochondrial membranes, was studied in the goldfish, *Carassius auratus*, (Böck *et al.*, 1980) and in the skate, *Raja erinacea*, (Stewart *et al.*, 1994).

Adapted to the habitat and specific needs of different species, the purine catabolism is quite variable, even between related groups. Urate oxidase was reported in some species, in which it behaves like a peroxisomal matrix soluble protein. It is the case of the mackerel, *Pneumatophorus japonicus*, and the yellow mackerel, *Trachurus trachurus*, (Noguchi *et al.*, 1979), as well as brown trout (Rocha *et al.*, 1999; 2003). Depending on the species, allantoinase and allantoicase can have different localizations but they have been described in peroxisomes of some fishes (Hayashi *et al.*, 1989b; Sakuraba *et al.*, 1996).

Fish hepatic peroxisomes also seem to have an important role in the metabolism of ROS. Besides the ubiquitous catalase, the antioxidant enzyme Cu,Zn-superoxide dismutase was detected in hepatic peroxisomes of the gilthead sea bream, (Pedrajas *et al.*, 1996).

Several other peroxisomal enzymes were already detected in a crescent number of fish species. Specifically in brown trout, spectrophotometric studies revealed the activities of catalase and the peroxisomal oxidases D-amino acid oxidase, acyl-CoA oxidase, glycolate oxidase and urate oxidase (Rocha *et al.*, 2001b; 2003; 2004; Resende *et al.*, 2005).

1.3. Peroxisome proliferator activated receptors (PPARs)

1.3.1. Peroxisome proliferation

Hess and coworkers reported for the first time the phenomenon of peroxisome proliferation in rat liver, after the administration of clofibrate, a hypolipidemic

drug (Hess *et al.*, 1965). Since then, many structurally diverse chemicals have been shown to induce a volume and number increase in hepatic peroxisomes of mammals, usually accompanied by induction of some peroxisomal enzymes activities, in particular the ones involved in lipid metabolism (Reddy and Lalwani, 1983; Rao and Reddy, 1987; Lock *et al.*, 1989). The same inducers also cause hepatomegaly, with cell hyperplasia and hypertrophy due to proliferation of the endoplasmic reticulum and peroxisomes (Grasso, 1993). Special interest has been devoted to this matter after the association of carcinogenesis with peroxisome proliferation in rodents (Reddy *et al.*, 1980). In the proliferation process, the β-oxidation enzymes activities enhance 20 to 30 fold (Reddy *et al.*, 1986; Nemali *et al.*, 1989), whereas catalase activity has only a 2 to 3 fold increase (Nemali *et al.*, 1989). This imbalance between the induction of H_2O_2 producing and degrading enzymes results in a marked increase of H_2O_2 levels, which could possibly be the cause for metabolic alterations that could lead to the emergence of mutagenic factors (Reddy and Rao, 1992). On the other hand, the suppression of hepatic apoptosis observed after administration of certain peroxisome proliferators, such as nafenopin, was also suggested to be a cause for hepatocarcinogenesis, once it generates an imbalance between cell gain and cell death (Bayly *et al.*, 1994).

Although the majority of the studies is focused on mammals, more attention is being recently paid to the proliferation process in aquatic animals (Fahimi and Cajaraville, 1995; Cajaraville *et al.*, 2003), since these can be used as indicators of aquatic environmental pollution. In fact, the industrial activity developed in the recent years has been overloading the environment with large quantities of proliferative chemicals, especially in watercourses. These include a wide range of structurally unrelated compounds, although certain characteristics may allow a group classification. Reddy and Lalwani (1983) established four classes of hepatic peroxisome proliferators: 1 – clofibrate and analogs, such as ciprofibrate, nafenopin and gemfibrozil; 2 – compounds structurally unrelated to clofibrate, like Wy-14643; 3 – plasticizers and related compounds, among which phthalate esters are the most important; 4 – dietary manipulations, such as a high fat diet. The first class comprises the chemicals generically termed hypolipidemic drugs, therapeutically used for control of hyperlipidemias. Several other classification tables were made that take into account the chemical structure of the compounds (Bentley *et al.*, 1993; Lake, 1995). Despite their great diversity, most possess an acidic function, which is in many cases a carboxyl group.

Data available on fish peroxisome proliferation include studies of several xenobiotics effect on salmonids. In rainbow trout, ciprofibrate injection was shown to increase hepatic peroxisomal volume density and some enzymes activities in a dose-related manner (Yang *et al.*, 1990). Depending on the concentration, water exposure to linuron and to atrazine increased peroxisome numbers in liver and proximal renal segments (Oulmi *et al.*, 1995a; 1995b). On primary cultured hepatocytes, clofibrate and ciprofibrate caused a strong dose-dependent induction of some enzymes, whereas gemfibrozil did not show any effect (Donohue *et al.*, 1993). Antagonic dose-related effects were observed with 4-chloroaniline. A low dose administered to juveniles decreased both peroxisome number and catalase activity, whereas a higher dose caused cluster formation and number increase on isolated hepatocytes (Braunbeck, 1993). Exposure time was also shown to produce different results. β-naphthoflavone injection caused an increase on the hepatic activity of superoxide dismutase until the second day and a subsequent decrease (Lemaire *et al.*, 1996). In Atlantic salmon isolated hepatocytes, the administration of clofibric acid and bezafibrate enhanced acyl-CoA activity (Ruyter *et al.*, 1997).

1.3.2. PPAR isotypes

In 1990, Isseman and Green isolated a mouse cDNA encoding for a protein that was activated by certain peroxisome proliferators based on its homology with several members of the nuclear hormone receptors family. In this way, it was named peroxisome proliferator activated receptor (PPAR). Two years later, three different PPARs were identified in the frog, *Xenopus laevis* (Dreyer *et al.*, 1992): **PPARα** (ortholog of the mouse isotype), **PPARβ** and **PPARγ**. These isotypes were soon detected in other mammals (Göttlicher *et al.*, 1992; Schmidt *et al.*, 1992; Jow and Mukherjee, 1995; Aperlo *et al.*, 1995). Due to homology discrepancies, PPARβ was called PPARδ or FAAR (fatty acid activated receptor) in rodents and NUC1 (nuclear hormone receptor 1) in humans.

Not being steroid, retinoid or thyroid hormone receptors, PPARs were initially grouped with another class of nuclear receptors termed “orphan receptors”, whose legitimate activating ligands had yet to be identified. However, it became evident that PPARs are activated by peroxisome proliferators and that many of the genes regulated by these compounds contain specific upstream recognition sites

called peroxisome proliferator response elements (PPREs), responsible for the effects of the proliferators (Schoonjans *et al.*, 1996). In order to bind DNA, the PPAR must form a heterodimer with another nuclear receptor, the 9-cis retinoic acid or retinoid X receptor (RXR). Thus, functional PPREs contain two copies of the core motif AGGTCA, separated by one nucleotide and are, therefore, called direct repeats 1 (DR1) (Tugwood *et al.*, 1992; Keller *et al.*, 1993). The functionality of all members of the nuclear hormone receptors family is achieved by a structural compartmentation in four domains that, besides this DNA binding region, include the ligand binding region, the transactivation functions and the dimerization interface (Lemberger *et al.*, 1996b; Escher and Wahli, 2000). Although these general features are common to all PPARs, the isotypes can vary substantially in terms of ligand specificity, physiological functions and tissue differential expression. Moreover, they are encoded by distinct single-copy genes located in different chromosomes (Jones *et al.*, 1995).

As a consequence of amino acid sequences divergence corresponding to the ligand binding domain (LBD) of the three PPAR isotypes, some **ligand specificity** is to be expected. There are, however, some ligands capable of complexing with more than one isotype (Fig.11). The first studies on PPAR activators have shown that low concentrations of the peroxisome proliferator Wy-14643 activate PPARα, but not PPARs β and γ (Kliewer *et al.*, 1994; Lehmann *et al.*, 1995). However, at higher concentrations, this hypolipidemic agent is able to activate PPARγ and even PPARβ (Lehmann *et al.*, 1995). It was also reported in these studies that PPARs β and γ are activated by linoleic acid and by the peroxisome proliferator LY-171883 (a leukotriene D_4 antagonist), respectively (Kliewer *et al.*, 1994) and that an antidiabetic compound of the thiazolidinedione class, rosiglitazone (BRL-49653), is a high affinity ligand for PPARγ exclusively (Lehmann *et al.*, 1995). Following research identified a prostaglandin J_2 metabolite and leukotriene B_4 as natural activating ligands for PPARs γ and α, respectively (Kliewer *et al.*, 1995; Devchand *et al.*, 1996). During the last decade, several studies have ascribed many other ligands for the PPAR isotypes, either exogenous or endogenous (reviewed by Corton *et al.*, 2000). In general, PPARα is preferentially activated by exogenous peroxisome proliferators like fibrates, phthalate esters and leukotriene D_4 antagonists and by endogenous fatty acids and certain eicosanoids like 8(S)- hydroxyeicosatetraenoic acid (HETE). PPARβ is better activated by fatty

acids, especially the polyunsaturated, like γ-linoleic acid. Curiously, the hypolipidemic drug bezafibrate and two nonsteroidal anti-inflammatory drugs are potent activators of this isotype. Antidiabetic thiazolidinediones almost exclusively activate PPARγ and the nonsteroidal anti-inflammatory drugs are preferential ligands for this isotype, which is also activated by mono- and polyunsaturated fatty acids and several eicosanoids (Corton *et al.*, 2000).

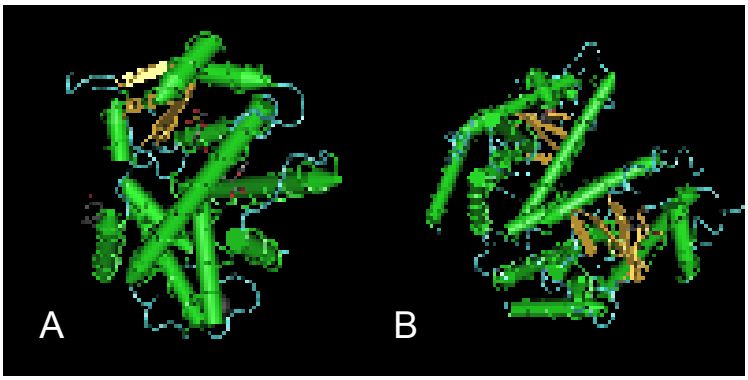


Fig. 11 – Crystal structure of the ligand binding domain from human PPARα (A) and PPARγ (B) in complex with the agonist Az 242. Taken from www.ncbi.nlm.nih.gov/.

Ligand specificity among PPARs reflects the involvement of each isotype in particular **functions** and the analysis of the corresponding target genes, when identified, can be a valuable clue to elucidate the role of PPARs. In this way, it became evident that PPARα is involved in fatty acid oxidation (Dreyer *et al.*, 1992; Tugwood *et al.*, 1992; Zhang *et al.*, 1992; Lee *et al.*, 1995) and also plays an important role in the down-regulation of inflammatory responses (Devchand *et al.*, 1996). The assignment of a specific role for PPARβ has not been an easy work, partially due to its ubiquitous tissue expression. It was inferred that this isotype may be involved in general cellular functions, such as membrane lipid synthesis and turnover (Braissant and Wahli, 1998). Interestingly, a stronger expression of PPARβ is observed during development, which may indicate a possible role of this receptor in cell differentiation (Braissant and Wahli, 1998). In addition, an elevated PPARβ expression was observed in the digestive tract of adult rat, where a high rate of cell renewal and differentiation occurs (Braissant *et al.*, 1996). More recently, it was also suggested an involvement of PPARβ in osteoclastic bone resorption (Mano *et al.*, 2000) and adipocyte function (Peters *et al.*, 2000). PPARγ was shown to be a potent regulator of terminal adipocyte

differentiation (Tontonoz *et al.*, 1994a; Chawla *et al.*, 1994). Furthermore, the inhibition of both macrophages activation and inflammatory cytokines production by PPARγ ligands suggest a role in inflammation control for this isotype (Ricote *et al.*, 1998; Jiang *et al.*, 1998).

As expected, **tissue distribution pattern** is different for each isotype, as well as for distinct species. In *X. laevis* both PPARα and PPARβ display an ubiquitous pattern of expression (Dreyer *et al.*, 1993), but in rodents PPARα is more expressed in liver, kidney, heart, stomach mucosa, duodenum mucosa and brown adipose tissue (Kliewer *et al.*, 1994; Braissant *et al.*, 1996; Lemberger *et al.*, 1996a), whereas PPARβ is abundantly expressed in most of the tissues except in the liver, where it is weakly expressed, and in smooth muscle, where it is not detected (Kliewer *et al.*, 1994; Braissant *et al.*, 1996). PPARγ is less expressed in general, being restricted to fat body and mesonephros in *X. laevis* (Braissant *et al.*, 1996). In rodents, adipose tissue seems to be the major site of PPARγ expression (Tontonoz *et al.*, 1994b), although lower levels of expression have been also detected in non-adipose tissues, such as spleen, duodenum mucosa and retina (Braissant *et al.*, 1996).

The **mechanisms of action** of PPARs are not yet clearly understood, but there is evidence that they are modulated by cofactors, which can either repress (corepressors) or enhance (coactivators) their transcriptional activities. It has been generally accepted that, if there are no ligands available, nuclear receptors might be bound to corepressors that prevent their transcriptional activity, sometimes enabling them to repress specific genes. After ligand binding, the nuclear receptors undergo conformational changes, which result in corepressor dissociation and coactivator binding, with subsequent activation of gene transcription (reviewed by Moras and Gronemeyer, 1998; Gelman *et al.*, 1999; Tan *et al.*, 2005).

None of the cofactors described so far seems to be specific for PPARs or a particular PPAR isotype. For example, the PPAR binding protein (PBP) was isolated on the basis of its association with the LBD of PPARγ (Zhu *et al.*, 1997) and it was further demonstrated in the same work that it also binds to $TR\beta_1$ (thyroid hormone receptor β_1), RARα (retinoic acid receptor α), and RXRα (retinoic X receptor α). Another example is the p300 group of proteins, which clearly

stimulate mouse PPARα transcriptional activity (Dowell *et al.*, 1997) and also coactivate a number of other nuclear receptors (Chakravarti *et al.*, 1996). Additionally, it was shown that one of the p300 related proteins interacts with the PPARγ LBD in 3T3-L1 cells (Mizukami and Taniguchi, 1997), although a positive effect on PPARγ transcriptional activity was not clearly established.

1.3.3. Fish PPARs

On account of medical and pharmacological problems and interests, studies on PPARs have concentrated essentially on mammal species but, during the last few years, environmental concerns have lead to an increasing interest on aquatic organisms PPARs. Among teleost fish, the zebrafish, *Danio rerio*, was the first species with all PPAR subtypes cloned (Escrivá *et al.*, 1997; Robinson-Rechavi *et al.*, 2001). The Atlantic salmon, *Salmo salar*, PPARγ (Ruyter *et al.*, 1997; Andersen *et al.*, 2000) and PPARβ (Robinson-Rechavi *et al.*, 2001) were cloned, although sequences from its PPARα have not been published. PPARγ from the plaice, *Pleuronectes platessa*, was first isolated in 1998 (Leaver *et al.*, 1998) and recently all of the plaice PPAR subtypes were cloned and characterized, as well as PPARs of the gilthead sea bream, *Sparus aurata*, (Leaver *et al.*, 2005) and of the sea bass, *Dicentrarchus labrax* (Boukouvala *et al.*, 2004). The turbot, *Scophtalmus maximus*, PPARβ was also cloned (Robinson-Rechavi *et al.*, 2001).

The bioinformatic analysis of the complete genome of the pufferfish, *Fugu rubripes*, revealed the existence of single orthologs of human PPARβ and PPARγ genes and two orthologs of human PPARα gene (Maglich *et al.*, 2003). PCR analysis showed that all PPAR genes were broadly expressed in the pufferfish, contrasting with the expression pattern of the human genes, where only the PPARβ isotype is abundantly expressed.

The tissue and cellular distribution of PPARs in zebrafish organs was assessed by immunohistochemistry (Ibabe *et al.*, 2002). PPARα is mainly expressed in hepatocytes, enterocytes, pancreas, renal proximal tubules and intestinal smooth muscle and serosa. PPARβ has a broader expression and is present in hepatocytes, bile ducts, pancreas, enterocytes, intestinal smooth muscle and serosa, renal tubules and glomeruli, spleen lymphocytes, epidermis, skeletal muscle and gonads. A weak expression of PPARγ is seen in pancreas and

intestinal smooth muscle and serosa, and a very weak staining is detected in enterocytes, lymphocytes and female gonads. Hepatocytes, epidermis and spermatogonia present a very weak PPARγ expression in some specimens. As to PPARs cellular distribution, most of the signal is cytoplasmic, except for hepatocytes, where some nuclear labelling for PPARs α and β is also detected (Ibabe *et al.*, 2002).

More recently, an immunohistochemistry study of PPARs expression was also made on the various hepatic cell types of gray mullet, *Mugil cephalus,* a very abundant species in European estuaries and coasts, used as sentinel for aquatic pollution studies (Ibabe *et al.*, 2004). In this species, immunolabeling for PPARα is found mostly in melanomacrophages, hepatocyte nuclei, sinusoidal cells and connective tissue surrounding bile ducts, and is barely detectable in hepatocyte cytoplasm and bile duct epithelium. Melanomacrophages show strong positivity for PPARβ, whereas sinusoidal cells and connective tissue around bile ducts are weakly stained. PPARβ staining is barely detected in hepatocyte cytoplasm and nuclei, and is negative in bile ducts epithelium. PPARγ expression is very weak, being restricted to melanomacrophages. A trypsin pre-treatment for antigen retrieval enhances the signal, which becomes weak or barely detectable in connective tissue surrounding bile ducts, sinusoidal cells and hepatocytes (Ibabe *et al.*, 2004).

The possible effects of known PPARα and PPARγ ligands on the expression of these isotypes were investigated in an *in vitro* model of zebrafish primary hepatocyte culture (Ibabe *et al.*, 2005). The specific PPARα ligand 8(S)-HETE, the specific PPARγ ligand prostaglandin J_2 and the peroxisome proliferator clofibrate were then shown to induce the expression of both PPAR isotypes, measured as immunolabeling for PPARs per cell.

Besides the environmental value of toxicological studies, knowledge on fish PPARs is also of economic interest. Taking into account the role of these receptors on lipid metabolism regulation, and the importance of high fat diets and lipid deposition on farmed fish (Tocher, 2003; Diez *et al.*, 2007), this has been an issue of increasing research, specially in aquaculture species.

1.4. References

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2. OBJECTIVES

Objectives

Studies on peroxisomes of aquatic species have been emerging in the last decade. Due to increasing evidence pointing to the relationship between environmental pollutants and peroxisome proliferation in fish and molluscs, this subject has been raising interest in the scientific community working in environmental toxicology and biomarkers development. Nevertheless, the current information about fish peroxisomes and PPARs is yet limited.

Considering the involvement of PPARs in peroxisomal metabolism regulation, and the modifications induced by estrogenic compounds on fish peroxisome morphology and biochemistry, as referred in the General Introduction, it is probable that PPARs may somehow be implicated in this process. By the other hand, they may be also involved in the seasonal peroxisomal changes demonstrated in trout. Therefore, the general purpose of our study was to enrich the knowledge on fish peroxisomes, by better understanding the mechanisms of physiological and structural regulation of brown trout peroxisomes by estrogenic compounds, identifying and clarifying the possible roles of PPARs and estrogen receptor in peroxisome dynamics. The specific objectives delineated to accomplish this task were:

1. To identify the different PPAR isotypes genes through partial sequenciation and comparison with other species isotypes, and to establish their organ expression pattern.
2. To quantify the variation in the hepatic levels of PPARs mRNAs in the different stages of the annual breeding cycle and in both genders.
3. To investigate the variation in the expression of peroxisomal enzymes regulated by estradiol.
4. To study the dynamics of morphological and metabolic alterations induced by estradiol in hepatic peroxisomes and to investigate the role of the estrogenic receptor in this process.

3. IDENTIFICATION AND ORGAN EXPRESSION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTORS IN BROWN TROUT (*SALMO TRUTTA* F. *FARIO*)

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Summary

Although widely studied in mammals, little information about fish peroxisome proliferator activated receptors (PPARs) is yet available. As a baseline for future studies, the three PPAR isotypes were identified in brown trout (*Salmo trutta* f. *fario*) and their organ distribution pattern was established. The cDNA fragments encoding PPARs α, β and γ were amplified by PCR, and the deduced sequences of the corresponding peptides were compared with other species sequences. Both the 183 amino acid sequence from PPARα and the 103 amino acid sequence from PPARβ shared high levels of homology with the corresponding peptides of other fishes and terrestrial vertebrates, whereas PPARγ 108 amino acid sequence showed much less similarity with non-fish PPARγ. According to both semi-quantitative RT-PCR and real-time RT-PCR, PPARα mRNA predominates in white muscle, heart and liver and PPARβ is more expressed in testis, heart, liver, white muscle and trunk kidney. PPARγ was only detected in trunk kidney and liver by real-time RT-PCR and also in spleen by semi-quantitative RT-PCR. PPARβ seems to be the most strongly expressed isotype, whereas PPARγ shows a much weaker global expression.

Introduction

Peroxisome proliferator activated receptors (PPARs) are a family of hormone receptors implicated in a collection of fundamental biological processes, mainly related to lipid metabolism regulation. Three PPAR isotypes – α, β and γ – encoded by separate genes and showing different tissue distribution patterns have been identified. In mammals, different physiological functions have been attributed to each isotype (for a review, see [1]). PPARα is mainly involved in regulation of lipid oxidation. This receptor regulates the expression of peroxisome proliferator responsive genes, including a number of genes implicated in peroxisomal β-oxidation pathway [2]. Additionally, a role in controlling inflammatory response has been suggested [3]. Because of its ubiquitous organ expression, no specific functions have been definitely assigned to PPARβ. It is thought that this receptor is implicated in basic cellular functions, such as lipid synthesis and turnover, and also in cell differentiation [4]. Moreover, some information suggests that this isotype plays an important role in osteoclastic bone resorption [5] and adipocyte function [6]. Finally, it has been shown that PPARγ is a key transcription factor involved in adipocyte differentiation [7,8] and it is also implicated in the inflammatory response [9,10].

All nuclear hormone receptors share a common structural and functional organization [11,12]. The A/B domain, located in the least conserved N-terminal region, is responsible for ligand-independent transactivation activity, comprising the activation function 1 (AF-1) element. The DNA-binding C domain (DBD) is highly conserved. A variable hinge region, the D domain, allows conformational changes of the protein. The activation function 2 (AF-2) element is situated in the C-terminal ligand binding E/F domain (LBD), responsible for ligand-dependent transcriptional activity [11,12].

To date, PPAR isotypes have been identified and cloned in several vertebrate species, such as primates [13], rodents [14], amphibians [15], birds [16] and some fish. The three isotypes have been cloned in zebrafish, *Danio rerio* [17,18]. PPARβ was cloned in Atlantic salmon, *Salmo salar* and turbot, *Scophthalmus maximus* [18]. PPARγ was cloned in Atlantic salmon [19,20] and European plaice *Pleuronectes platessa* [21]. Recently, Ibabe and coworkers made an immunohistochemical study of the PPAR isotypes expression and distribution

patterns in several organs of *D. rerio* [22] and in liver of gray mullet, *Mugil cephalus* [23].

To enrich the current knowledge about these receptors in fish and to establish baseline data for future toxicological and peroxisomal metabolic regulation studies with *S. trutta*, we cloned the three PPAR isotypes in this species and evaluated their organ distribution pattern using both semi-quantitative RT-PCR and real-time RT-PCR. This was made for the first time in a fish species. The relevance of our study is reinforced by recent data showing PPARs involvement in chemically induced peroxisome proliferation in zebrafish [24].

Materials and methods

Chemicals

Guanidine thiocyanate, phenol, chloroform, isopropanol and diethylpyrocarbonate were obtained from Sigma Chemical Co. (St. Louis, USA). Thermoscript™ RT-PCR System, Platinum® Taq DNA Polymerase, dNTP Mix (PCR Grade), PCR primers, Deoxyribonuclease I (Amplification Grade) and TOPO TA Cloning® Kit were purchased from Invitrogen Life Technologies (Carlsbad, USA). InViSorb™ DNA Extraction Kit and InViSorb™ Spin Plasmid Midi Kit were purchased from InViTek (Berlin, Germany). Yeast Extract and Tryptone Peptone (DIFCO) were obtained from Becton Dickinson Microbiology Systems (Sparks, USA). EcoR I (Recombinant) was obtained from New England Biolabs Inc. (Beverly, USA). 2x iQ SYBR Green Supermix was from Bio-Rad (Hercules, USA).

Fish and sampling

Adult male brown trout (*Salmo trutta* f. *fario* Linnaeus, 1758) specimens weighing in average 800 g were obtained from a governmental aquaculture facility, where they were fed a commercial diet following routine procedures. Collection of the animals by random net fishing took place in early October, when gonads are maturing towards the breeding season (December/January). Liver, head and trunk

kidney, heart, spleen, testis, blood, and white muscle were locally harvested, quickly frozen in liquid nitrogen and stored at -80° C until RNA processing.

Isolation of RNA and cDNA synthesis

Total RNA was isolated by the guanidine thiocyanate/phenol chloroform extraction method adapted from [25] and converted to cDNA by Thermoscript™ and an oligo $(dT)_{20}$ primer, according to the manufacturer's protocol.

PCR amplification and sequencing of PPAR genes

cDNA preparations from liver and trunk kidney were used in PCR amplifications. Oligonucleotide PCR primers were designed according to known evolutionary conserved sequences of certain PPAR domains, available at NCBI (www.ncbi.nlm.nih.gov). PCR primers were as follows:

PPARα Fw: 5'-ATCTTCCACTGCTGCCAGTG-3'

PPARα Rv: 5'-TACATGTCCCTGTAGAT-3'

PPARβ Fw: 5'-ACCCTGTGGCAAGCAGA-3'

PPARβ Rv: 5'-AACTTGGGCTCCATGAT-3'

PPARγ Fw: 5'-GGATGCCCCAAGTGGAGA-3'

PPARγ Rv: 5'-CTCTGTGAACCCAGGGAT-3'

PCR was performed according to the manufacturer's instructions and run on a Biometra® Personal Cycler (Biometra, Göttingen, Germany) PCR device. PPARγ was amplified for 7 min at 94° C, followed by 30 cycles of 94° C for 30 s, 54° C for 30 s and 72° C for 30 s, with a final extension step of 7 min at 72° C. After gel extraction following the manufacturer's instructions, the resulting PCR fragment was reamplified by a second run. PPARα was amplified for 7 min at 94° C, followed by 35 cycles of 94° C for 30 s, 54° C for 1 min and 30 s and 72° C for 30 s, with a final extension step of 7 min at 72° C and reamplified by a second

run. PPARβ was amplified for 7 min at 94° C, followed by 30 cycles of 94° C for 30 s, 54° C for 30 s and 72° C for 30 s, with a final extension step of 7 min at 72° C. All PCR products were purified from 1% agarose gels and cloned into *E. coli* through pCR®II-TOPO plasmid vector. After extraction of selected plasmids from positive colonies, according to the manufacturer's protocol, the presence of PCR fragments was confirmed by EcoR I restriction followed by agarose gel electrophoresis. These plasmids were then sequenced by MWG-Biotech AG (Ebersberg, Germany). Partial sequences of PPARs were compared using MultAlin (prodes.toulouse.inra.fr/multalin/multalin.html) [26] and BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Semi-quantitative RT-PCR

In a first approach to know the distribution pattern of PPARs, semi-quantitative RT-PCR was made. Before conversion to cDNA, RNA samples were subjected to DNase I treatment, according to the manufacturer's protocol. All PCR primers were designed in order to generate products with the same length for the different genes (236 bp). The oligonucleotide sequence of each primer was chosen within the sequence of the whole fragment previously obtained:

PPARα Fw: 5'-TTCAGCGACATGATGGAGCC-3'

PPARα Rv: 5'-CAGTTTCTGCAGCAGATTGG-3'

PPARβ Fw: 5'-AGGAGATAGGGGTACACGTG-3'

PPARβ Rv: 5'-CAGGAACTCCCGGGTCACAA-3'

PPARγ Fw: 5'-TGTCTGTCCTACCACAGAC-3'

PPARγ Rv: 5'-CGGAACTGGATGCGGCGGA-3'

The following procedure was used to perform semi-quantitative PCR: 236 bp fragments of PPARs α, β and γ were amplified by a programme of 94° C for 5 min, 40 cycles of 94° C for 30 s, 58° C for 30 s and 72° C for 30 s and a final extension step of 72° C for 10 min. The products were visualized on a 0.9% agarose gel containing 0.0025% ethidium bromide and the band densities were

determined by digital image analysis, using a Kodak Digital Science DC 120 Zoom Digital Camera and Kodak Digital Science 1D Image Analysis Software, version 3.5 for Windows (Eastman Kodak Co., New York, USA). Analysis results are expressed in an arbitrary semi-quantitative scale, using β-actin as housekeeping gene.

Real-time RT-PCR

After the preliminary data of semi-quantitative RT-PCR, relative levels of PPAR isotypes mRNAs were quantified by real-time RT-PCR analysis using the iCycler iQ real-time PCR detection system (Bio-Rad). Two µl of each cDNA sample were added to a reaction mix containing 2x iQ SYBR Green Supermix and 200 nM of each primer (as above), making a total volume of 25 µl per reaction. A template free negative control was included for each set of primers. All reactions were run on duplicate in a 96-well plate. The cycling profile was equal to the one used in the semi-quantitative RT-PCR. A melting curve was generated for every PCR product to confirm the specificity of the assays and a dilution series was prepared to check the efficiency of the reactions. The PPARs gene expression in each organ was normalized to β-actin and calculated as the fold increase relative to an arbitrary fold increase of 1 (for blood PPARα, which had the lowest value). The fold increase was calculated as $2^{-(O\Delta Ct - B\Delta Ct)}$, where B is blood, O is another organ and ΔCt is the delta threshold cycle (Ct) between the gene of interest and β-actin.

Results

PPARs partial gene sequences

The cDNA and corresponding predicted amino acid sequences resulted from the amplification experiments have been deposited to GenBank with the accession numbers DQ139936, DQ139937 and DQ139938.

cDNA amplification with oligonucleotide primers for PPARα resulted in a 590 bp product as expected, which encoded 183 amino acid residues after exclusion of the primers (Fig. 1A). A comparison between this amino acid sequence and the corresponding PPARα sequences of the teleosts goldfish (*Carassius auratus*) and

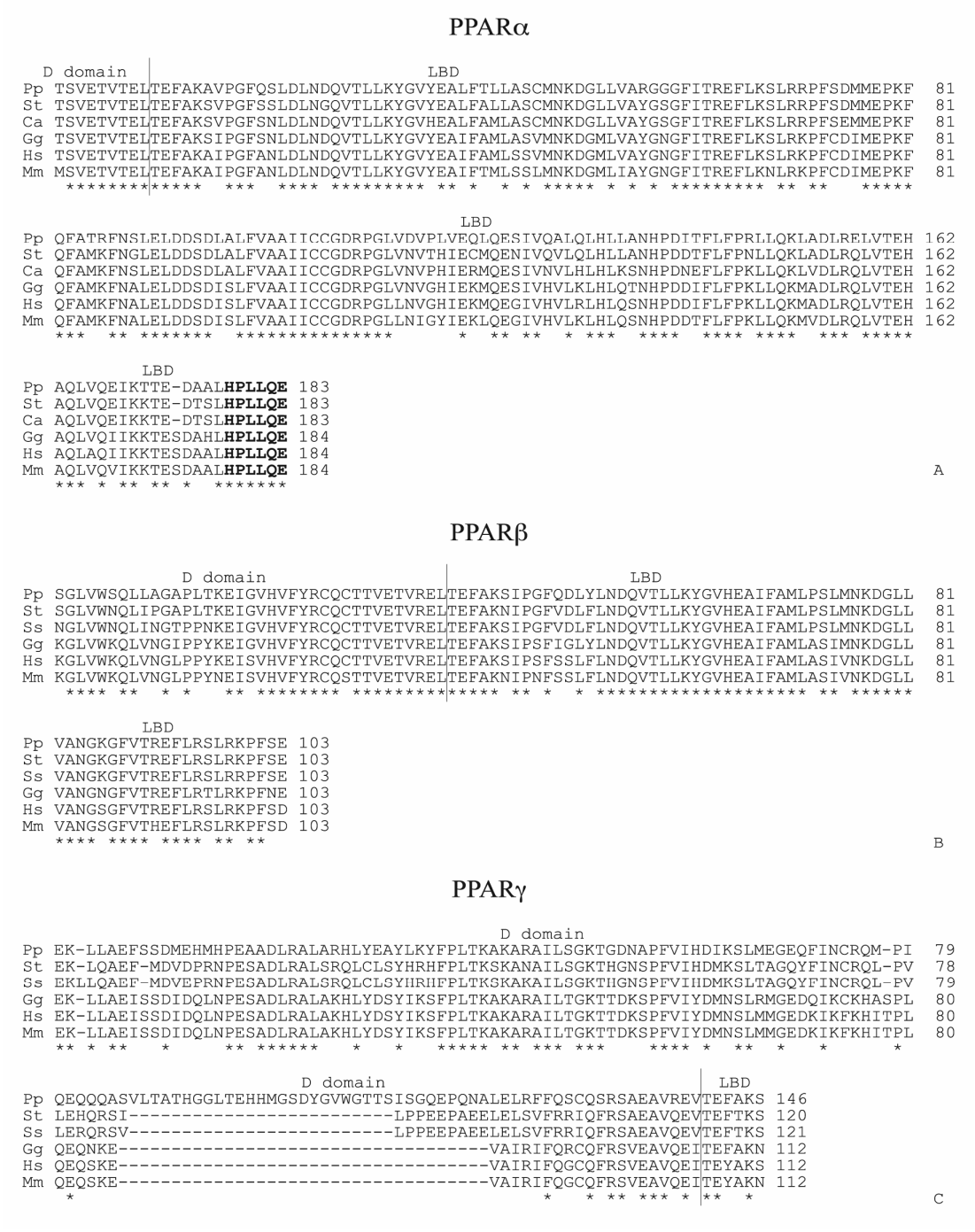

Fig. 1 – Comparison of the predicted amino acid sequences for PPARs of *Salmo trutta* f. *fario* (St) with the corresponding sequences of other species. Pp – *Pleuronectes platessa*, Ca – *Carassius auratus*, Gg – *Gallus gallus*, Hs – *Homo sapiens*, Mm – *Mus musculus*, Ss – *Salmo salar*. LBD – ligand binding domain. Asterisks indicate identical residues. The gaps introduced allow the maximization of identities. Bold letters correspond to part of the AF-2 motif of PPARα. All sequences are available in NCBI (www.ncbi.nlm.nih.gov). The new sequences have been deposited to GenBank with the accession numbers DQ139936, DQ139937 and DQ139938.

P. platessa showed 90% and 87% identity, respectively (Fig. 1 A). High levels of identity were also obtained with the chicken, *Gallus gallus*, human, *Homo sapiens*, and mouse, *Mus musculus*, PPARα (84%, 82% and 78%, respectively). This region comprises the last few residues of the D domain and almost the entire E/F domain (LBD). Part of the highly conserved motif HPLLQEIYR, that constitutes the AF-2 element required for the ligand-dependent transcription, is in the C-terminal portion (Fig. 1 A). All fish PPARα lack one S residue near the C-terminus in relation to terrestrial vertebrates.

PCR amplification of PPARβ originated a product of the expected size of 344 bp, corresponding to 103 amino acid residues (Fig. 1B). This sequence belongs to part of the D domain and part of the LBD. Identities with sequences of other species are high: 94% identity with *P. platessa*, 93% with *S. salar*, 84% with *G. gallus* and *H. sapiens*, and 82% with *M. musculus*. The number of amino acid residues is maintained among the species.

The sequence of the 401 bp fragment generated by PCR amplification of PPARγ encoded 120 amino acid residues located mostly in the D domain (Fig. 1C). This portion of the *S. trutta* protein shared 97% identity with *S. salar* PPARγ and less (under 52%) with other species. The *S. trutta* PPARγ contains an insertion of 10 residues in the D domain that is not present in terrestrial vertebrates. Because in *P. platessa* the insertion is much larger, the identity of the sequenced portion of the protein between these fishes is only of 46%.

PPARs tissue expression

The expression pattern of *S. trutta* PPAR isotypes in heart, liver, head and trunk kidney, spleen, testis, blood, and white muscle, after semi-quantitative RT-PCR, is shown in Table 1. The results were based on densitometric band analysis, with β-actin as housekeeping gene, as shown in Fig. 2.

According to this method, PPARα predominates in heart and white muscle, being less expressed in head kidney and blood. PPARβ is present in all organs tested. It is particularly abundant in heart, liver, trunk kidney, testis, and white muscle. Finally, PPARγ is the most selectively expressed PPAR, being only detected in trunk kidney and, to a lesser extent, in liver and spleen. Regarding the relative

overall expression of all PPAR isotypes, the data suggest a slight predominance of PPARβ over PPARα, being PPARγ the less expressed one.

Table 1 – Relative expression levels of PPAR genes in *S. trutta* organs by semi-quantitative RT-PCR

	Organs							
	Heart	Liver	Head kidney	Trunk kidney	Spleen	Testis	Blood	White muscle
PPARα	++	+	±	+	+	+	±	++
PPARβ	++	++	+	++	+	++	+	++
PPARγ	–	±	–	+	±	–	–	–

Values were obtained by densitometric band analysis, using β-actin as reference gene. Expression levels: undetectable (–), barely detectable (±), weak expression (+), strong expression (++).

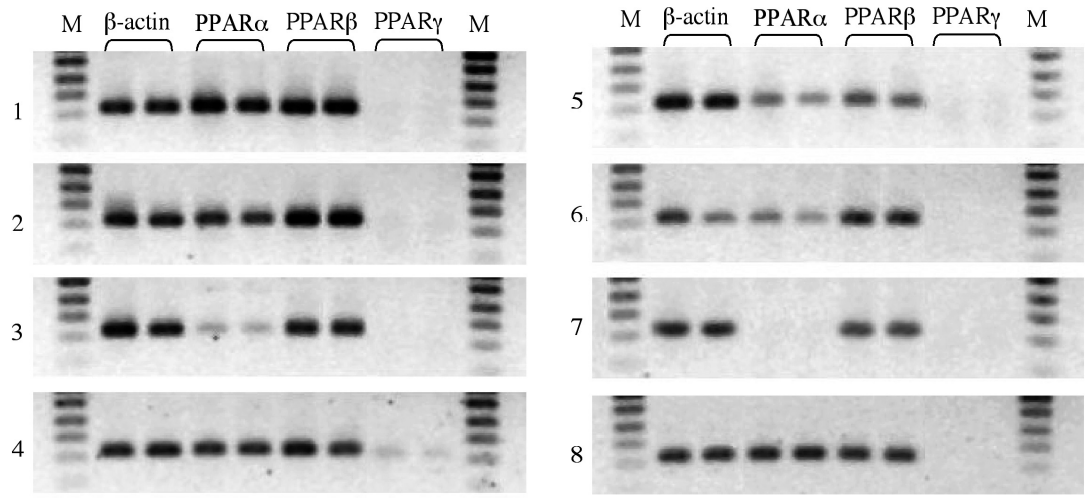

Fig. 2 – Expression of PPAR isotypes in *S. trutta* organs, after RT-PCR. Each PCR reaction was made in duplicate (brackets). M – DNA ladder. 1 – heart, 2 – liver, 3 – head kidney, 4 – trunk kidney, 5 – spleen, 6 – testis, 7 – blood, 8 – white muscle.

Table 2 shows the relative expression levels of PPARs generated by real-time RT-PCR. PPARα expression is stronger in white muscle, followed by heart and liver.

PPARβ maintained its pattern and PPARγ was not detected in spleen, but the overall distribution pattern is the same.

Table 2 - Relative expression levels of PPAR genes in *S. trutta* organs by real-time RT-PCR

	Organs							
	Heart	Liver	Head kidney	Trunk kidney	Spleen	Testis	Blood	White muscle
PPARα	3,822	1,176	6	315	64	181	1	14,263
PPARβ	15,159	15,093	1,065	8,128	2,508	85,644	5,696	12,946
PPARγ	0	6	0	24	0	0	0	0

All values were established in relation to blood PPARα level, as detailed under Materials and Methods.

Discussion

In parallel with a significant reduction of liver peroxisome volume, it was shown that the activity of some liver peroxisomal enzymes is also reduced in vitellogenic *S. trutta* females, which have high levels of estradiol [27,28]. Considering PPAR involvement in peroxisomal metabolism regulation [1], these receptors may somehow be implicated in the peroxisomal alterations observed in *S. trutta*. Our current studies on this species PPARs are aimed to contribute for understanding their role in the cited structural and functional peroxisomal dynamics.

Since 1990, PPARs have been studied in an increasing number of species, especially in mammals. In the last few years their existence has also been verified in some fishes [17-23]. From analyses of the cDNA and predicted amino acid sequences identities between the products cloned in *S. trutta* and those described in other species, we conclude that the novel sequences from *S. trutta* are, indeed, PPARs α, β and γ.

The fragments of PPARs α and β analysed show a high level of identity among species, whereas the fragment of PPARγ is the less conserved with evolution. This may be due to the difference in the regions compared: PPARγ comprises a great

part of the D domain, which is recognized as the less conserved, whereas PPARα shows almost the entire LBD and PPARβ parts of both domains. When comparing the same region domains of PPARs α and β and the same regions of PPARs β and γ, we note that PPARβ presents the highest level of identity among species and PPARγ the lowest (Fig. 1). Curiously, the PPARγ of the salmonids *S. trutta* and *S. salar* have a 10 amino acid residues segment in the D domain that is not present in the terrestrial vertebrates protein, with the pleuronectid *P. platessa* having an even larger insertion. This feature seems to be characteristic of fish PPARγ and stretches the variability of the D domain, not only in sequence but also in length. Once more, PPARβ shows great homogeneity among species, having the same number of amino acid residues in all the sequenced extension of the protein. In PPARα, this number varies only by one residue between fish and non-fish proteins.

The analysis of tissue distribution of the different PPAR isotypes is often useful for getting clues or for better understanding their physiological roles. The quantitative real-time RT-PCR assays generally confirmed the results obtained with semi-quantitative RT-PCR.

In *S. trutta*, PPARβ appears in all organs tested, predominantly in testis, heart, liver, white muscle and trunk kidney. This is in accordance with the notion that PPARβ is generally widespread. Additionally, we found that in *S. trutta* the PPARβ isotype showed the strongest overall expression, in accordance with what happens in Sprague-Dawley rats [29] and in the fish *D. rerio* [22]. Nevertheless, when *S. trutta* and rodents are compared, some discrepancies in the distribution pattern of PPAR isotypes can be noted. The most striking differences are the PPARα expression in liver and the PPARγ expression in spleen, both very high in rodents [29-31] and much weaker in *S. trutta*. These discrepancies may be due to differences in organ constitution and functions among species; it is known that mammals and fish differ in hepatic lobulation and metabolic zonation, and even in the spatial arrangement of hepatocytes [32].

In relation to PPARα, our data indicate that it is more expressed in white muscle, heart and liver than in trunk kidney, spleen and testis, and also that it is very weakly expressed in both head kidney and blood. The data suggests a role of PPARα in regulating the peculiar metabolic demands of the trout cardiomyocytes, and of the white (oxidative, type I) muscle fibres. In accordance with this idea, a PPARα role in regulating rodent cardiac fatty acid oxidation and glucose oxidation

rates has been well characterized [33], and PPARα activation was recently shown critical in cardiac mitochondrial cardiolipin biosynthesis [34]. As to white muscle, it is proven for several models that PPARα is implicated in the development of type I muscle fibres, also playing an important role in changes in their mitochondria content [35]. Moreover, oxidative use of carbohydrate and lipid does occur in trout white muscle [36] and so, PPARα involvement and significant expression is logically expected.

In rodents, high levels of PPARγ are found essentially in adipose tissue and immune system organs [29,31], in consonance with its physiological role in adipocyte differentiation and regulation of inflammation. It has also been detected, though to a lesser extent, in rodent intestine and kidney cells [29–31]. The immune related functions in teleosts are primarily assigned to kidney, spleen, and also thymus [37]. In fact, in *S. trutta* we have found the strongest PPARγ expression in trunk kidney, followed by a weak expression in liver but virtually none in spleen and head kidney. In *M. cephalus*, PPARγ presents a higher expression in melanomacrophages as detected by immunohistochemistry [23] and, according to our observations in healthy *S. trutta,* trunk kidney is the richest organ in melanomacrophages, which can explain the higher expression of PPARγ in this organ. Moreover, PPARγ was not detected in kidney tubules and glomeruli of *D. rerio* [22]. Since it was recently found that PPARγ expression in monocytes and macrophages is related with anti-inflammatory activities [38], it is admissible that the increased expression we found in trunk kidney can be related to local regulatory mechanisms of melanomacrophages. Considering that PPARγ was not detected in rodent liver [29–31], the very weak expression of PPARγ in *S. trutta* liver can be mainly related to the presence of some melanomacrophages in this organ. A relatively low pool of these cells is present in the liver of healthy salmonids, but they appear in larger numbers in the liver of other fish or in pathological conditions [32,39].

In conclusion, novel genes for *S. trutta* were cloned and identified as PPARs α, β and γ. For the first time, a parallel semi-quantitative and quantitative study of PPARs mRNAs was made in fish, showing the relative expression of these receptors in several organs. PPARα predominates in white muscle, heart and liver; PPARβ is more expressed in testis, heart, liver, white muscle and trunk kidney; PPARγ was only quantified in trunk kidney and liver. PPARβ slightly predominates over PPARα, and PPAR γ seems to be the least expressed isotype.

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4. Seasonal and Gender Variation of Peroxisome Proliferator Activated Receptors Expression in the Liver of Brown Trout

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Summary

PPAR isotypes α, β and γ have been previously identified in the teleost brown trout (*Salmo trutta* f. *fario*) and their organ distribution pattern established. Being the liver a vital metabolic organ presenting expression of all isotypes and also knowing that estrogens somehow interact with PPARs, it was relevant to conduct a study on the expression variation of these receptors in the liver, along the annual reproductive cycle and in both genders. According to real-time RT-PCR, PPARα mRNA expression in females was significantly higher in May and lower in September than in all other seasons. No significant variation was observed along the year in males. A significant difference between genders occurred in May, when PPARα expression was higher for females. PPARβ expression showed little variation along the reproductive cycle in females but, in males, it was significantly higher in December than in the other seasons. It was not significantly different between genders in any season. PPARγ was differently expressed between February and September, and also between February and December, for females. As to males, it differed between February and all the other seasons. In both cases, the expression in February was always higher. No significant differences were observed between genders. The study proved our hypothesis that PPARs gene expression varies along the year. Moreover, the PPARα expression in females followed the same annual variation pattern as peroxisome volumes and enzyme activities, and an inverse pattern relatively to their annual plasma estradiol levels previously determined, which contributes to the idea that PPARα is under estradiol modulation and that a cross-talk between this receptor and the estrogen receptor possibly exists.

Introduction

In 1990 a member of the nuclear hormone receptors family was cloned from the mouse (Isseman and Green, 1990) and shown to be activated by a class of chemicals which cause peroxisome proliferation. It was, thus, termed peroxisome proliferator-activated receptor (PPAR). Soon after this discovery, this and two additional related receptors sharing significant homology in their encoded DNA and protein structures were cloned from other species (Dreyer *et al.*, 1992; Göttlicher *et al.*, 1992; Schmidt *et al.*, 1992; Jow and Mukherjee, 1995; Aperlo *et al.*, 1995). The mouse isotype was called PPARα, being the other ones termed PPARβ, sometimes referred to as PPARδ, FAAR or NUC1, and PPARγ.

The three PPAR isotypes are encoded by separate genes and show different tissue distribution patterns. Although each isotype is involved in specific physiological functions, this class of receptors is generally implicated in processes related to lipid metabolism regulation (reviewed by Escher and Wahli, 2000; Corton *et al.*, 2000). These hormone receptors are inactive in the absence of either the retinoid X receptor (RXR) or the respective ligands for any of them. After ligand binding, PPAR heterodimerizes with RXR, without which it can not bind DNA and subsequently activate gene transcription (Kliewer *et al.*, 1992; Gearing *et al.*, 1993).

Not being PPARs ligands, estrogens are known to somehow regulate PPARs expression. For example, PPARα mRNA in rat skeletal muscle was shown to be up-regulated by 17β-estradiol alone (Campbell *et al.*, 2003). Furthermore, there is evidence for a possible cross-talk between the ovarian hormones in order to regulate PPARα mRNA levels, once treatment with progesterone in combination with estradiol suppressed the up-regulation effect of this hormone (Campbell *et al.*, 2003). On the other hand, estradiol was reported to down-regulate PPARγ mRNA expression in a mesenchymal mouse cell line (Dang *et al.*, 2002). Immunohistochemical studies in zebrafish isolated hepatocytes showed that the percentage of PPARγ positive cells was lower under the effect of estradiol, but the expression of PPARγ per cell was increased by this estrogen (Ibabe *et al.*, 2005).

Stereological studies made in transmission electron microscopy in brown trout liver demonstrated that peroxisome individual and total volumes were reduced in late vitellogenic females (Rocha *et al.*, 1999). Accordingly, the activity of some

peroxisomal enzymes, especially of urate oxidase, was considerably decreased in those animals (Rocha *et al*., 2001; 2004b; Resende *et al*., 2005). Knowing that estradiol highest levels occur in late vitellogenesis and prespawning (Fostier *et al*., 1983; Rocha *et al*., 2001; 2004) and that this hormone interacts with PPARs, which regulate peroxisomal lipid metabolism, it became pertinent to investigate if PPARs are involved in the morphological and metabolic changes induced by estradiol in brown trout hepatic peroxisomes. As a first approach, the PPARs expression variation pattern along the year in liver of sexually mature brown trout specimens of both genders was quantified by both semi-quantitative and real-time RT-PCR. We hypothesized that PPARs expression changes along the breeding cycle, and that the variations probably differ among genders, in accordance with the suspected regulatory role of sex steroids.

Materials and methods

Chemicals

Guanidine thiocyanate, phenol, chloroform, isopropanol, diethylpyrocarbonate (DEPC), agarose and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, USA). DNase I (Amplification Grade), Thermoscript™ RT-PCR System, Platinum® Taq DNA Polymerase, dNTP Mix (PCR Grade) and PCR primers were purchased from Invitrogen Life Technologies (Carlsbad, USA). The real-time PCR fluorophore mix, 2x iQ SYBR Green Supermix, was from Bio-Rad (Hercules, USA).

Animals and sampling

Three-year-old brown trout (*Salmo trutta* f. *fario* Linnaeus, 1758) specimens were obtained from a governmental aquaculture facility. They had been maintained under natural photoperiod and temperature range in outdoor tanks with continuous running water and they were hand fed a commercial diet, following established routine production procedures. Six females and six males were collected by random net fishing at major periods of their annual reproductive cycle, as observed in Portugal: postspawning (February), early vitellogenesis

(May), late vitellogenesis (September) and prespawning (December). After fish dissection under deep anaesthesia (using a solution of ethylene-glycol monophenyl ether, 1 ml/L), livers were snap frozen in liquid nitrogen and stored at -80º C until further processing. Gonads were inspected and weighted after removal, for confirming the reproductive stage. Values of the morphometric parameters of the pool from which fishes were sampled are shown in Table 1.

RNA isolation and cDNA synthesis

All materials used for RNA preparation were treated with 0.1% DEPC and/or sterilized for 4 hours at 200º C. Liver total RNA was isolated by the guanidine thiocyanate/phenol chloroform extraction method adapted from Chomczynski and Sacchi (1987). RNA concentration and purity were assessed by measurement of optical density at 260 nm and its integrity was checked by visualization of rRNA bands after agarose gel electrophoresis. To remove contamination with residual DNA, all samples were subjected to DNase I treatment following the manufacturer's instructions. RNA was then reverse transcribed to cDNA with oligo $(dT)_{20}$ primers by Thermoscript™ RT-PCR System, according to the manufacturer's protocol.

Semi-quantitative RT-PCR

All PCR primers were designed in order to generate products with the same length for the different genes (236 bp). The oligonucleotide sequences of the primers were chosen based on known sequences of brown trout PPARs previously obtained (Batista-Pinto *et al.*, 2005) and were as follows:

PPARα Fw: 5'-TTCAGCGACATGATGGAGCC-3'

PPARα Rv: 5'-CAGTTTCTGCAGCAGATTGG-3'

PPARβ Fw: 5'-AGGAGATAGGGGTACACGTG-3'

PPARβ Rv: 5'-CAGGAACTCCCGGGTCACAA-3'

PPARγ Fw: 5'-TGTCTGTCCTACCACAGAC-3'

PPARγ Rv: 5'-CGGAACTGGATGCGGCGGA-3'

Semi-quantitative RT-PCR was performed with Platinum® Taq DNA Polymerase and dNTP Mix, according to the manufacturer's specifications and run on a Biometra® Personal Cycler (Biometra, Göttingen, Germany) PCR device. The cycling profile consisted of a programme of 94º C for 5 min followed by 40 cycles of 94º C for 30 s, 58º C for 30 s and 72º C for 30 s and a final extension step of 72º C for 10 min. The products were visualized on a 0.9% agarose gel containing 0.0025% ethidium bromide and the band densities were determined by digital image analysis, using a Kodak Digital Science DC 120 Zoom Digital Camera and Kodak Digital Science 1D Image Analysis Software, version 3.5 for Windows (Eastman Kodak Co., New York, USA). The values for each group of animals correspond to the means obtained for the respective cDNA samples and are expressed in an arbitrary semi-quantitative scale, normalized relative to β-actin from the same cDNA samples.

Real-time RT-PCR

Quantitative real-time RT-PCR analysis on PPAR isotypes mRNAs expression levels was performed on an iCycler iQ real-time detection system (Bio-Rad). Two µl of each cDNA sample were added to a reaction mix containing 2x iQ SYBR Green Supermix and 200 nM of each primer (the same used in the semi-quantitative study), making a total volume of 25 µl per reaction. The PCR protocol was as described above. All reactions were run on duplicate in a 96-well plate, including a template free negative control for each set of primers. Melting curves (92 steps of 10 s, from 50.0 to 95.5º C, with a temperature gradient increase of 0.5º C per step) were generated for PCR products to confirm the specificity of the assays and to check the occurrence of primer dimmers. A dilution series was also prepared to test the efficiency of PCR amplifications. Threshold cycle (Ct) values for each cDNA sample were normalised to the Ct values of the internal standard β-actin for the same sample. The fold increase of 1 was arbitrarily attributed to the first sample treated (S1, a February male). The values for the other samples were calculated as the fold increase relative to this one, using the formula $2^{-(S\Delta Ct - S1\Delta Ct)}$, where S is the sample in question and ΔCt is the delta threshold cycle (Ct) between this sample and β-actin.

Statistics

As the basic premises for a parametric analysis were not met, quantitative real-time RT-PCR data were analysed using non-parametric tests: Kruskal-Wallis ANOVA by Ranks, to detect seasonal effects and Mann-Whitney U test, to test specific differences between groups. Results were presented as the median of six animals per group. Contrarily to the other PPARs, PPARγ results could be normalized and homogenized by square root transformation, which allowed an additional parametric analysis by the Neuman-Keuls post hoc test. When $p \leq 0.05$ the differences were considered statistically significant.

Results

Fish morphometric parameters

Mean values of the morphometric parameters body weight, standard length, hepatosomatic index and gonadosomatic index of the total pool of animals contributing to this study are presented in Table 1.

Semi-quantitative RT-PCR

The expression levels of female and male brown trout liver PPAR isotypes along the year, after semi-quantitative RT-PCR, are shown in Table 2. These results were based on densitometric band analysis, with β-actin as housekeeping gene, as shown in Fig. 1.

Semi-quantification suggested a predominance of PPARα in early vitellogenic females and prespawning males over the other groups, which had a moderate expression. PPARβ female expression was also moderate, showing no apparent variation along the year. As to males, this isotype was abundant in the prespawning season, with a moderate expression in the other seasons. PPARγ was not detected in early vitellogenic females, being hardly detected in the other female groups. Postspawning males had a very weak PPARγ expression, with

undetectable levels in the other seasons. According to this quantification method, the relative overall expression of PPARα and PPARβ was identical and way far above PPARγ expression.

Table 1 – Fish morphometric parameters

Month	Gender	Weight (g)	Standard length (cm)	HSI	GSI
February	Female	626.6 (0.19)	36.1 (0.06)	1.505 (0.18)^{A}	0.632 (0.22)^{Aa}
	Male	682.0 (0.09)	35.7 (0.04)	1.459 (0.24)	1.637 (0.52)^{Ab}
May	Female	770.7 (0.19)^{A}	38.6 (0.08)^{A}	1.320 (0.17)	0.810 (0.17)^{Ba}
	Male	796.0 (0.13)^{A}	38.2 (0.05)^{A}	1.265 (0.26)	0.202 (0.28)^{Bb}
September	Female	526.2 (0.16)^{B}	33.2 (0.05)^{B}	1.655 (0.10)^{A}	12.202 (0.24)^{Ca}
	Male	564.7 (0.16)^{B}	33.6 (0.06)^{B}	1.337 (0.22)	5.341 (0.20)^{Cb}
December	Female	614.9 (0.22)	34.4 (0.07)^{B}	0.953 (0.32)^{B}	19.150 (0.09)^{Da}
	Male	758.6 (0.29)^{A}	36.4 (0.09)	1.226 (0.25)	3.560 (0.24)^{Db}

Results are presented as mean (CV), where CV = coefficient of variation = standard deviation / mean; HSI = hepatosomatic index = 100 x liver weight / animal weight; GSI = gonadosomatic index = 100 x gonad weight / animal weight. Different upper case superscript letters represent differences among months within a gender ($p \leq 0.05$). Diferent lower case superscript letters represent differences between genders within each month ($p \leq 0.05$).

Table 2 – Relative seasonal expression levels of PPAR genes in brown trout liver by semi-quantitative RT-PCR

	Females				Males			
	February	May	September	December	February	May	September	December
PPARα	+	++	+	+	+	+	+	++
PPARβ	+	+	+	+	+	+	+	++
PPARγ	±	-	±	±	±	-	-	-

Values were obtained by densitometric band analysis, using β-actin as reference gene. Expression levels: undetectable (-), barely detectable (±), moderate expression (+), strong expression (++). February: postspawning, May: early vitellogenesis, September: late vitellogenesis, December: prespawning.

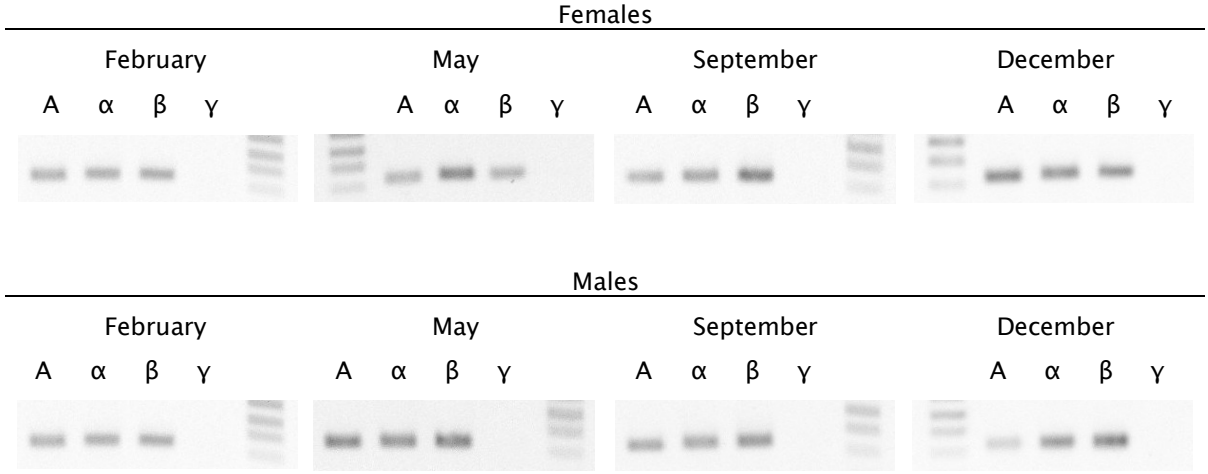

Fig. 1 – Expression of PPAR isotypes in brown trout liver after semi-quantitative RT-PCR, as described under Materials and methods. Example of one animal per group. A – β-actin, α – PPARα, β – PPARβ, γ – PPARγ. All PCR fragments were designed to have 236 bp.

Quantitative real-time RT-PCR

Appropriate statistic treatment was applied to quantitative real-time RT-PCR data on PPAR isotypes mRNAs expression levels. For PPARα and PPARβ analysis, a non-parametric approach was followed, once the data sets failed to be normalized. However, an additional parametric analysis confirmed the same variations. As to PPARγ, both normalization and homogenization of variances were achieved by square root transformation, allowing an additional parametric treatment which reinforced the results. The results of the non-parametric analysis for all isotypes are depicted in Fig. 2.

In females, PPARα expression was significantly higher in early vitellogenesis and lower in late vitellogenesis than in the other seasons. Conversely, the males did not show a significant variation in its expression level along the reproductive cycle. Gender differences were only significant during early vitellogenesis, when females had higher levels of expression.

The expression of the β isotype along the year showed little variation in females. On the other hand, in males it increased significantly in prespawning, decreasing in the following season to the previous levels and remaining low for the rest of the year. No significant differences were detected between genders in any season.

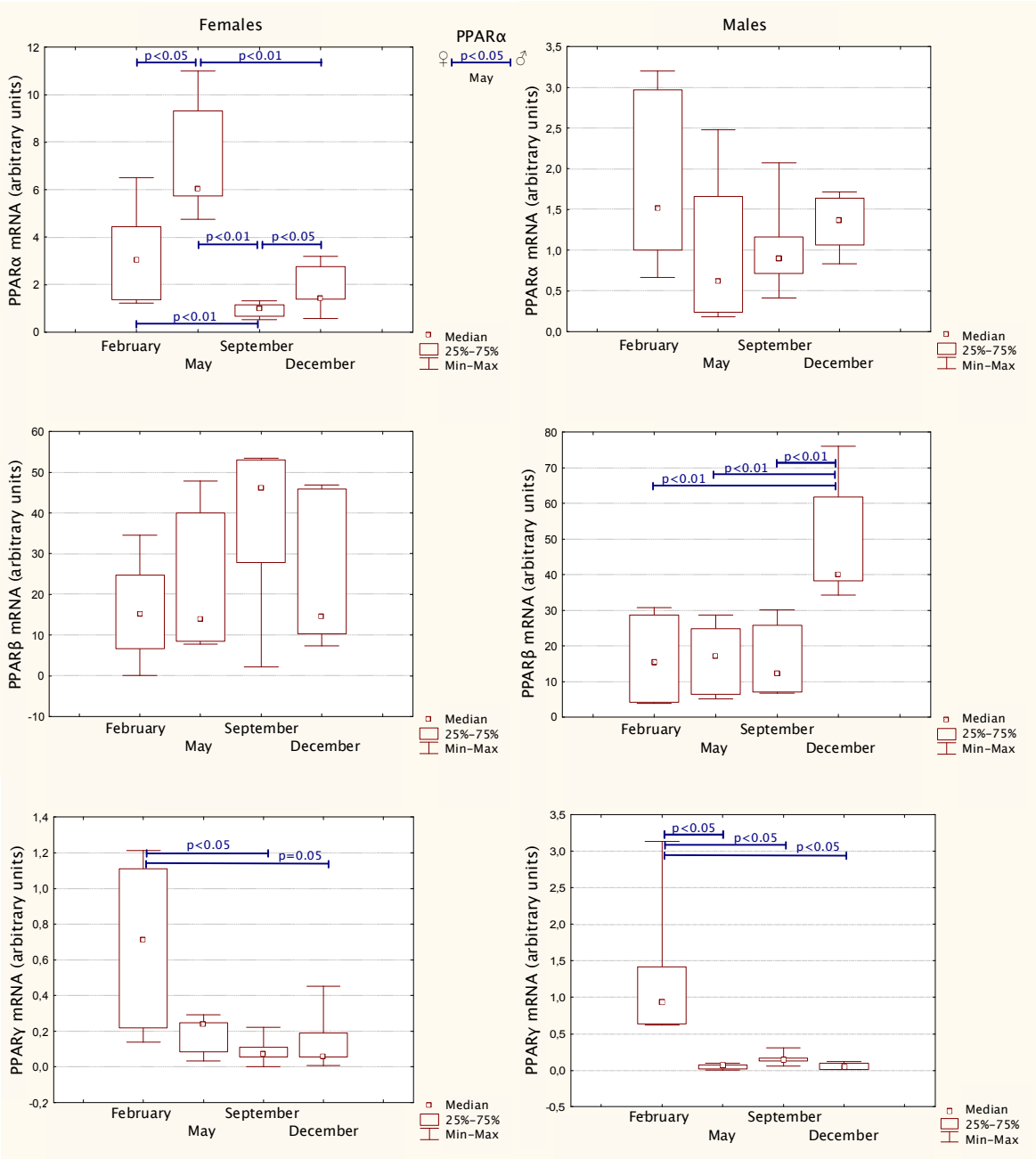

Fig. 2 – Expression of PPAR isotypes in brown trout liver after quantitative real-time RT-PCR, as described under Materials and methods. February: postspawning, May: early vitellogenesis, September: late vitellogenesis, December: prespawning.

PPARγ expression in female groups was significantly higher in postspawning than in late vitellogenesis and prespawning, with a marginal difference in the latter. Considering the significant results of the Neuman-Keuls parametric pairs test, the difference between postspawning and early vitellogenesis expressions can be faced as on the verge of significance. The expression level of this receptor was

also elevated in postspawning males, which differed significantly from all the other groups. Differences between genders were not significant in any season.

Discussion

A previous work reported the identification and organ distribution pattern of PPARs α, β and γ in brown trout (Batista-Pinto *et al.*, 2005). According to this study, the general highest expression of PPARs was found in the liver. This is, indeed, a crucial metabolic organ, with a complex multifunctional activity. Synthesis of the egg yolk proteins precursor vitellogenin after estrogen stimulus is among liver assignments (Tata, 1976). It was therefore pertinent to investigate the expression of PPARs in the liver depending on gender and reproductive stage, even more by knowing that these receptors may be also influenced by estrogens (Dang *et al.*, 2002; Campbell *et al.*, 2003; Ibabe *et al.*, 2005).

PPARs expression quantification was made by real-time RT-PCR using cDNAs of six females and six males per season. A previous semi-quantitative RT-PCR assay using the same cDNA samples was included in the study for comparative purposes. Although this technique does not allow detection of minor expression variations and can not be considered as accurate as the quantitative method, it still provides a reliable qualitative measurement of the expression levels pattern (reviewed by Freeman *et al.*, 1999; Halford *et al.*, 1999). Hein and coworkers (2001) analyzed comparatively four different mRNA quantification techniques and came to the conclusion that semi-quantitative non-competitive RT-PCR follows the results of real-time RT-PCR, the most accurate and advantageous method, being more sensitive than Northern blot analysis and ELISA. However, for best results it is essential that the product yield be measured during the exponential phase of the amplification reaction (Freeman *et al.*, 1999; Halford *et al.*, 1999). In this work, only small discrepancies were observed in the results obtained by both methods, strengthening the inferences. Besides the lower sensitiveness inherent to the semi-quantitative process, one can admit that the number of PCR cycles used had reached the plateau phase, reason why the PPARβ expression was not much above α isotype expression, as observed with the quantitative technique. On the other hand, when the quantitative assays were made, a sixth cDNA sample per group was added in order to improve the statistic analysis, which could have

led to minor differences in the results. The following discussion will consider real-time RT-PCR data, only.

Early vitellogenic brown trout females showed a significantly higher PPARα expression level than all the other groups. During late vitellogenesis, its level dropped considerably. Testosterone and estradiol measurements along the reproductive cycle of brown trout females have shown that, like other salmonid females, they have minimum steroids levels in postspawning and early vitellogenesis and very high levels in late vitellogenesis and prespawning (Rocha *et al.*, 2001; 2004b). Thus, the annual patterns of PPARα and these steroids are negatively related to each other. Additionally, a reduction in peroxisomal parameters, namely organelle volumes, and catalase, urate oxidase and palmitoyl-CoA oxidase activities in brown trout liver, was previously reported by our group in late vitellogenic and prespawning females (Rocha *et al.*, 1999; 2001; 2004b; Resende *et al.*, 2005). The observed PPARα expression variation is in consonance with these facts, once this receptor is involved in peroxisome proliferation events and in the regulation of the peroxisomal enzymes involved in lipidic metabolism (Dreyer *et al.*, 1992; Lee *et al.*, 1995). A study of the total lipid balance made in female rainbow trout showed that there is a preferential utilisation of mobilised lipids for energy production during early vitellogenesis and for storage in the oocytes during late vitellogenesis (Nassour and Léger, 1989). In this way, a decrease in PPARα expression between early and late vitellogenesis could be expected.

In early vitellogenesis, when lipids are used to energy purposes rather than to ovary growth, PPARα expression was significantly higher in females than in males. No significant changes in the expression of this receptor along the year were seen in males. Our preceding morphological results also indicated that males undergo a less pronounced variation in peroxisome parameters than females (Rocha *et al.*, 1999). Interestingly, the gender influence on PPARα expression variation and intensity observed in brown trout is different from the observed in rat. Several studies reported that male rats are more responsive than females to the general effects of peroxisome proliferation (Gray and de la Iglesia, 1984; Yamada *et al.*, 1991; Sundseth and Waxman, 1992). Accordingly, the rat hepatic basal levels of both PPARα mRNA and protein were shown to be higher in males (Jalouli *et al.*, 2003). Future studies on peroxisome proliferators in brown trout should take season and gender differences into account, once the baseline expression level of this receptor can influence the response intensity.

In brown trout PPARα expression, the variabilities observed in each season were generally higher in males, which naturally lack estradiol regulation. Variability among females in late vitellogenesis and in the following season was very low, probably due to the constancy of hormonal stimuli. In postspawning, however, females initiate the process towards early vitellogenesis at different rates, which can be a factor of increasing variability. These facts seem to be another indication that PPARα is somehow regulated by estrogens.

In the mouse, the expression of urate oxidase, a peroxisomal enzyme not regulated by PPARs, was not induced by several peroxisome proliferators which activated PPARα (Hurst and Waxman, 2003). Curiously, in female brown trout liver, urate oxidase activity and PPARα expression variations followed the same pattern, suggesting that both genes may be regulated by estradiol. More studies on fish urate oxidase are needed to clarify the regulation of this enzyme.

The constitutive expression of PPARβ is patent in the female annual cycle. Its levels were the most elevated among the three isotypes and did not show any significant variation along the year. Significant expression differences between genders were not detected in any season, but the males, curiously, presented a significantly higher level of PPARβ in prespawning than in the other seasons. These patterns are not related with prior observations either on plasma steroid levels or on peroxisomal parameters (Rocha *et al.*, 1999; 2001; 2004b; Resende *et al.*, 2005). Although the physiological functions of this PPAR isotype are not clearly determined yet, a role in epidermal cell proliferation and wound healing has been stated (Peters *et al.*, 2000; Tan *et al.*, 2004). In a competition for breeding, spawning salmonid males develop an aggressive strategy, which leads to violent male-to-male fighting (Fleming 1996; Petersson *et al.*, 1999). Thus, an elevated expression of PPARβ is compatible with an increased require for tissue repair and healing processes during prespawning, and we hypothesize that the expression increase we actually saw in the liver can result from more general triggering signal(s) that would increase the expression of this PPAR in several organs. Such signal(s) could well be, for example, the steep rise of 11-ketotestosterone and the presence of sexual pheromones that are known for long to occur at this period in salmonids and other fishes (Scott *et al.*, 1980; Liley and Stacey, 1983). Facing the present data, it is possible that, in fish, specific gender dependent functions for PPARβ will be revealed if deeper studies are made.

The expression patterns and levels of PPARγ along the breeding cycle were shown to be quite similar between genders, with both postspawning males and females having significantly higher levels than the other groups. No graphic correlation was observed between PPARγ expression and peroxisomal parameters or plasma steroid level patterns (Rocha *et al.*, 1999; 2001; 2004b; Resende *et al.*, 2005). With specific known functions in particular cell types, such as the adipocytes (Tontonoz *et al.*, 1994; Chawla *et al.*, 1994), and in the immune system tissues (Ricote *et al.*, 1998; Jiang *et al.*, 1998), PPARγ is generally the least expressed isotype. Its expression in brown trout was previously detected in liver and trunk kidney, and thought to be associated with the immune response (Batista-Pinto *et al.*, 2005). In a wide range of vertebrate species the immune system undergoes seasonal changes, which can be related to environmental factors such as temperature, photoperiod and food availability (Nelson and Demas, 1996), or can be driven by endogenous endocrine rhythms (Zapata *et al.*, 1992). Seasonal variations in immunocompetence associated with breeding are commonly known. Testosterone has been shown to exert an immunosuppressive effect in salmonids (Slater and Schreck, 1993; 1997). Moreover, the degree of immunosupression was proven to be significantly affected by season (Slater and Schreck, 1993). In sexually mature brown trout males, the frequency of ectoparasitic infestation was shown to be increased (Richards and Pickering, 1978; Pickering and Christie, 1980). Elevated plasma levels of the stress hormone cortisol are also known to reduce the immune response in brown trout and other salmonids (Tripp *et al.*, 1987; Pickering, 1989). However, the mechanism by which this receptor may be involved in the immune response is not clear yet. On the other hand, together with dramatic gonadal changes in both genders (Table 1), hepatic remodelling is known to occur along the breeding cycle of fishes, including salmonids, and especially in females (van Bohemen *et al.*, 1981; Srivastava and Saxena, 1996; Tripathi and Verma, 2004). One of the mechanisms involved in hepatic turnover, which may include or not changes in hepatosomatic index, is the macrophage mediated phagocytosis of apoptotic hepatocytes (Dini *et al.*, 2002). Thus, the salmonid life style and the events associated with the spawning season may be related to the increased PPARγ levels detected in postspawning. Moreover, the remarkable similarity between the variation patterns observed in both genders may point to the existence of a strong gender-independent regulation mechanism.

In conclusion, the annual expression pattern exhibited by PPARα in the liver of brown trout females have shown a close resemblance with the seasonal variation patterns of the morphological and biochemical peroxisomal parameters, and a negative relation with the plasma steroid levels previously observed (Rocha *et al.*, 1999; 2001; 2004b; Resende *et al.*, 2005). On the contrary, the expression variations of PPARα in males, as well as PPARs β and γ isotypes in both genders, are not related with those parameters. These facts support the hypothesis that hepatic peroxisomes are under a regulatory mechanism involving not only estradiol and estrogen receptor, but also PPARα.

Regardless of the considerations above made, one must bear in mind that the expression of a particular mRNA is not an absolute indication of the quantities and effects of the corresponding protein because regulatory mechanisms do exist which can act at translational and/or post-translational levels. Future work involving the study of PPAR proteins themselves is not to be discarded.

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5. Catalase in Brown Trout: Gene Identification, Organ Transcription Pattern and Seasonal Expression in the Liver

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Summary

Catalase is a peroxisomal marker enzyme with antioxidant functions. This ubiquitous and highly conserved protein has been characterized in numerous species, from prokaryotes to eukaryotes. To extend our studies on seasonal and gender variations of peroxisomes and their enzymes and on the hypothesized regulation by sex steroids, the gene encoding for catalase was identified in brown trout (*Salmo trutta* f. *fario*). A partial cDNA sequence was amplified by PCR and the predicted peptide sequence analysed and compared with corresponding sequences of other species. Catalase mRNA expression pattern in selected organs was determined by real-time RT-PCR, as well as hepatic catalase expression variation in both genders along the reproductive cycle. The analysis of an 83 amino acid peptide deduced from a 252 bp long nucleotide sequence showed high homology with fragments of the same protein from other species, including several teleost fishes. Among the organs tested, this transcript was shown to be abundant in liver and blood, moderately expressed in testis, white muscle and trunk kidney, weakly expressed in heart and head kidney, and scarcely present in spleen. In females, hepatic catalase mRNA expression was significantly higher in February and May than in September and December. The expression for males was nearly the same along the year. Regarding gender differences, significantly higher levels of expression were observed for males in December. We conclude that female seasonal variation pattern of hepatic catalase expression was seemingly coincident with its known seasonal activity and negatively related to the corresponding plasma estradiol levels previously determined, thus pointing towards a negative regulation of the enzyme by estrogens.

Introduction

Being responsible for the decomposition of hydrogen peroxide to oxygen and water, catalase plays an essential role, along with other enzymes, in protecting cells and organisms against the damaging effects of reactive oxygen species (ROS) and reactive oxygen intermediates (ROI), (Mackay and Bewley, 1989; Singh, 1997; Rudneva, 1999). It is particularly important in aerobic organisms and it has been widely described in bacteria and plants, and to some extent in fungi and animals (Klotz *et al.*, 1997). Reports of catalase sequences in aquatic animals are somewhat recent and include descriptions in the white shrimp, *Penaeus (Litopenaeus) vannamei*, (Tavares-Sánchez *et al.*, 2004) and in the zebrafish, *Danio rerio*, (Ken *et al.*, 2000; Gerhard *et al.*, 2000). These sequences present high similarity among vertebrates, not only at primary structure level, but also in what concerns to secondary, tertiary and quaternary structure. Mammalian catalase, which is usually confined to peroxisomes, consists of a heme containing homotetramer with a total molecular mass of approximately 240 kDa (Deisseroth and Dounce, 1970). Catalase protein characterization in aquatic organisms like the bullfrog, *Rana catesbeiana* Shaw, (Jang *et al.*, 2004), the white shrimp (Tavares-Sánchez *et al.*, 2004) and the zebrafish (Ken *et al.*, 2000) report an identical protein structure, sometimes with a lower molecular weight.

During the last few years, a great deal of attention has been given to the investigation of peroxisomes and peroxisomal enzymes. Besides a strong implication in human health due to their relationship with a class of severe diseases (Goldfischer *et al.*, 1973; Singh, 1997), peroxisomal enzymes and morphological parameters are also important tools to environmental pollution studies (Cajaraville *et al.*, 2003). Within this perspective, catalase has been very useful for morphometric characterization of peroxisomes (Orbea *et al.*, 1999a; 1999b; Krishnakumar *et al.*, 2004), being the classic marker enzyme for this organelle (Novikoff and Goldfischer, 1969; Fahimi, 1969). Detection of catalase enzyme activity is also documented in different tissues and cell types, developmental stages and annual cycle of many species (Schisler and Singh, 1987; Orbea *et al.*, 1999b; Cancio *et al.*, 1999). However, information on catalase gene expression is not so abundant. Besides plant tissues, there have been some studies in certain mammal organs like liver, brain or heart (Nemali *et al.*, 1988; Semsei *et al.*, 1991; Shi and Bekhor, 1994; Dieterich *et al.*, 2000). A tissue

expression pattern based on semi-quantitative RT-PCR was made for the white shrimp (Tavares-Sánchez *et al*., 2004), and for the mussel, *Mytilus galloprovincialis*, and the thicklip grey mullet, *Chelon labrosus*, (Bilbao *et al*., 2006).

The present work focus on catalase expression in brown trout: after gene identification from a partial sequence, a distribution pattern was made in eight organs, and the liver expression was established for both genders along the reproductive cycle. The seasonal study of a peroxisomal enzyme expression variation is integrated in a set of experiments designed with the purpose to investigate the influence of estrogenic compounds in the regulation of trout peroxisomes. The knowledge of catalase basal seasonal expression is also of special importance to toxicological studies, once the alterations in this enzyme are used as a bioindicator of environmental pollution.

Materials and methods

Chemicals

Guanidine thiocyanate, phenol, chloroform, isopropanol, diethylpyrocarbonate (DEPC), agarose and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, USA). DNase I (Amplification Grade), Thermoscript™ RT-PCR System, Platinum® Taq DNA Polymerase, dNTP Mix (PCR Grade) and PCR primers were purchased from Invitrogen Life Technologies (Carlsbad, USA). QIAquick PCR Purification Kit was purchased from QIAGEN (Hilden, Germany). The real-time PCR fluorophore mix, 2x iQ SYBR Green Supermix, was from Bio-Rad (Hercules, USA).

Fish and sampling

Three-year-old brown trout (*Salmo trutta* f. *fario* Linnaeus, 1758) specimens were obtained from a governmental aquaculture facility. The fish had been maintained in outdoor tanks with continuous running water under natural conditions of temperature and photoperiod and they were being fed a commercial diet, following routine production procedures. For the characterization of catalase and

the expression pattern study, two males were collected by random net fishing in early October, when gonads are maturing towards the breeding season (December/January). Liver, head and trunk kidney, heart, spleen, testis, blood, and white muscle were locally harvested, quickly frozen in liquid nitrogen and stored at -80º C until RNA extraction. For the seasonal study, seven males and seven females were collected in the same way at major seasons of their annual reproductive cycle, as observed in Portugal: postspawning (February), early vitellogenesis (May), late vitellogenesis (September) and prespawning (December). Following animal dissection under deep anaesthesia (using a solution of ethylene-glycol monophenyl ether, 1 ml/L), livers were immediately frozen in liquid nitrogen and stored at -80º C until further processing. For confirming the breeding status, gonads were inspected and weighted after removal. Values of the morphometric parameters of the pool from which fishes were sampled are shown in Table 1 of Chapter 4.

RNA isolation and cDNA synthesis

Materials used in RNA preparation were previously treated with 0.1% DEPC and/or sterilized for 4 hours at 200º C. Total RNA was extracted by the guanidine thiocyanate/phenol chloroform extraction method adapted from Chomczynski and Sacchi (1987). Concentration, quality and integrity of extracted RNA samples were assessed by spectrophotometric measure and electrophoretic visualization of rRNA bands. The samples were then treated with DNase I, according to the manufacturer's instructions, to remove residual contaminating DNA. The subsequent RNA (2 µg) conversion to cDNA was made with the Thermoscript™ RT-PCR System through the oligo $(dT)_{20}$ primer method, following the manufacturer's protocol.

PCR amplification and sequencing of the catalase gene

cDNA prepared from liver RNA was used in PCR amplification. Oligonucleotide PCR primers were designed according to known evolutionary conserved sequences of catalase from several species, available at NCBI (www.ncbi.nlm.nih.gov). Forward primer was 5'-CACTGATGAGGGCAACTGGG-3'

and reverse primer was 5'-CTTGAAGTGGAACTTGCAG-3'. This set of primers resulted in a 302 bp fragment. PCR reaction was performed according to the manufacturer's specifications and run on a Biometra® Personal Cycler (Biometra, Göttingen, Germany) PCR device. The amplification was accomplished by a programme of 5 min at 94° C, followed by 25 cycles of 94° C for 30 s, 58° C for 30 s and 72° C for 30 s, with a final extension step of 7 min at 72° C. Visualisation of PCR products on a 0.9% agarose gel containing 0.0025% ethidium bromide showed a single clean band. The resulting PCR product was then purified with QIAquick PCR Purification Kit following the manufacturer's instructions and sent for sequenciation through STABvida (Lisboa, Portugal). Partial sequence of catalase was compared with corresponding sequences of other species using MultAlin (prodes.toulouse.inra.fr/multalin/multalin.html) (Corpet, 1998) and BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

Quantitative real-time RT-PCR

Relative levels of catalase mRNAs in each organ were quantified by real-time RT-PCR analysis using the iCycler iQ real-time PCR detection system (Bio-Rad). The reaction mixture consisted of 23 µl of 2x iQ SYBR Green Supermix with 200 nM of each primer and 2 µl of cDNA sample. Cycling conditions were as described above. An additional programme of 92 steps for 10 s, with a temperature gradient increase of 0.5° C per step from 50.0° C to 95.5° C, generated a melting curve for PCR products to confirm the specificity of the assays and to check the occurrence of primer dimmers. A standard dilution series of cDNAs was also used to test the efficiency of the reactions. All assays were run on duplicate in a 96-well plate and included a template free negative control to assure the absence of contaminants. Threshold cycle (Ct) values for each cDNA sample were normalised to the Ct values of the internal standard β-actin for the same sample. The fold increase of 1 was arbitrarily attributed to the previously determined expression level of PPARα in the liver of a February male (S1). The values for the other samples were calculated as the fold increase relative to this one, using the formula $2^{-(S\Delta Ct - S1\Delta Ct)}$, where S is the sample in question and ΔCt is the delta threshold cycle (Ct) between this sample and β-actin.

Statistics

Although normalization of the results was possible with logarithmic transformation, homogeneity of variances could not be achieved. In this way, all data sets were analysed using the non-parametric tests: Kruskal-Wallis ANOVA by Ranks, to detect seasonal effects and Mann-Whitney U test, to test specific differences between groups. When $p \leq 0.05$ the differences were considered statistically significant.

Results

Partial gene sequence

The amplification of liver cDNA with oligonucleotide primers designed for catalase resulted in a 302 bp long fragment, as expected. Sequenciation in both strands allowed a consensus of 252 bp, after exclusion of the primers, which encoded for 83 amino acid residues, as determined by the correct reading frame. The alignment of this partial sequence with corresponding sequences from other teleosts showed high levels of identity (Fig. 1). Brown trout catalase was 90% identical to the European eel, *Anguilla anguilla*, catalase and 89% identical to the same sequence of the orange-spotted grouper, *Epinephelus coioides*, the rockbream, *Oplegnathus fasciatus*, the Atlantic cod, *Gadus morhua*, and the zebrafish, *Danio rerio*. Two sequences from terrestrial vertebrates were included in the alignment for comparison. Even with the mouse, *Mus musculus*, and the cow, *Bos taurus*, the identity level of this portion was as high as 88%. This region comprises several residues involved in the formation of α-helices and β-sheets in the mouse (Reimer *et al.*, 1994), and in the interaction with the heme group in the cow (Murthy *et al.*, 1981). The latter are conserved among species, whether the first slightly diverge (Fig.1).

Tissue expression

The relative expression levels of brown trout catalase in heart, liver, head and trunk kidney, spleen, testis, blood, and white muscle, as generated by RT-PCR, is

shown in Table 1. Catalase was present in all organs tested, being more expressed in liver and blood, followed by testis, white muscle and trunk kidney. Catalase expression was low in heart and head kidney and very low in spleen.

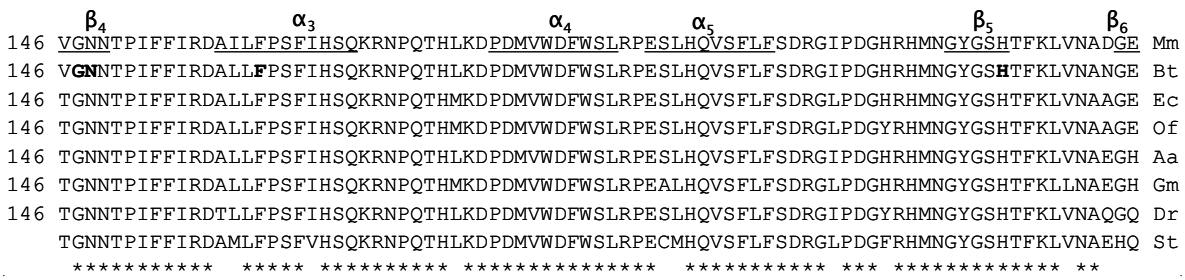

Fig. 1 – Alignment of the predicted amino acid partial sequence for catalase of brown trout and the corresponding sequences of other vertebrate species. Mm – *Mus musculus*, Bt – *Bos taurus*, Ec – *Epinephelus coioides*, Of – *Oplegnathus fasciatus*, Aa – *Anguilla anguilla*, Gm – *Gadus morhua*, Dr – *Danio rerio*, St – *Salmo trutta* f. *fario*. Asterisks indicate identical residues. The α-helices and β-sheets regions in the mouse sequence are underlined. Bold letters in the cow sequence correspond to the amino acid residues in contact with the heme. All sequences except the one for brown trout are available in NCBI (www.ncbi.nlm.nih.gov) with the accession numbers X52108.1, NM001035386, AY735009.1, AY734528.1, DQ493908.1, DQ270487.1, AJ007505, respectively.

Table 1 – Relative expression levels of catalase gene in brown trout by real-time RT-PCR

	Organs							
	Heart	Liver	Head kidney	Trunk kidney	Spleen	Testis	Blood	White muscle
Catalase	55	1,885	13	111	8	278	1,389	194

All values were established in relation to liver PPARα level of a February male, as detailed under Materials and methods.

Seasonal expression

The annual pattern of hepatic catalase expression levels in both genders after statistic treatment of quantitative real-time RT-PCR data is shown in Fig. 2.

Catalase mRNA expression in females was higher in postspawning and early vitellogenesis, then decreasing until prespawning. The differences were

significant between early vitellogenesis and both late vitellogenesis and prespawning, and also between postspawning and both late vitellogenesis and prespawning. No group variations along the reproductive cycle were observed for catalase expression in males. These evidenced a far greater interindividual variability than females, except in early vitellogenesis, when variabilities in both genders were similar. Differences between genders were significant only in postspawning, when catalase expression was higher in males.

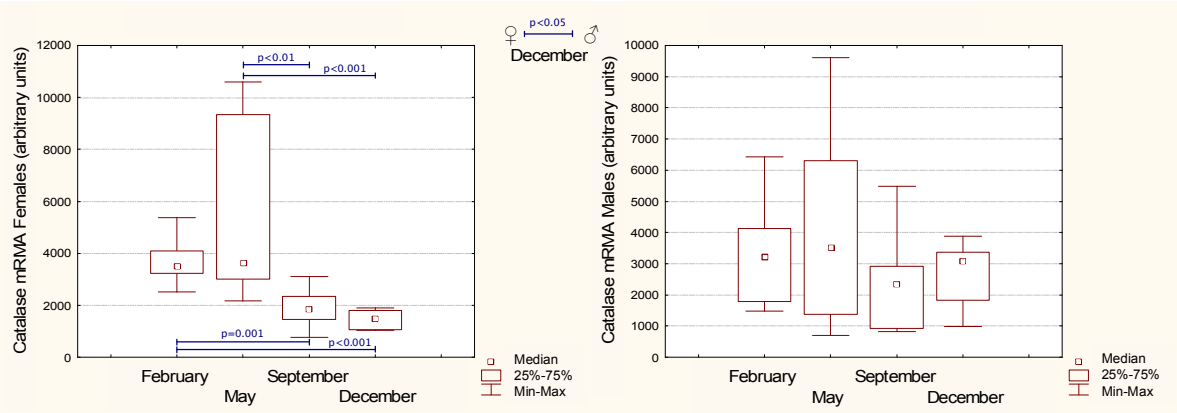

Fig. 2- Expression of hepatic catalase in brown trout after quantitative real-time RT-PCR, as described under Materials and methods. February: postspawning, May: early vitellogenesis, September: late vitellogenesis, December: prespawning.

Discussion

After sequenciation of the PCR fragment obtained with the primers designed for catalase, a multi alignment analysis was made that evidenced great identity between this partial sequence and the corresponding ones in other species. For now, the purpose of this procedure was to make sure that we were dealing with brown trout catalase mRNA rather than to sequence and characterize the whole gene in this species. The characterized region was, indeed, quite conserved (88 to 90% identity) among the studied species, not only in aquatic but also in terrestrial vertebrates. Considering the whole protein, the identity level between zebrafish and vertebrates such as man, guinea pig, rat, dog, pig, cow, mouse and frog ranges from 76 to 81% (Gerhard *et al.*, 2000; Ken *et al.*, 2000). Lower levels of identity arise when comparisons are made with other kinds of organisms, like the fruit fly (70%), nematodes (64%) and yeasts (51%) (Ken *et al.*, 2000). The most

divergent regions are the ones that enclose residues involved in the formation of α-helices 1, 6, 10, 12 and 13, and β-sheets 6 and 9 (Gerhard *et al.*, 2000). None of these regions is present in the 83 amino acids sequence obtained for brown trout, except part of the β_6-sheet. The last two residues of the trout sequence correspond to the first two of the β_6-sheet and are, in fact, not identical to the corresponding residues of the other species. Interestingly, the brown trout sequence contains several unique residues in positions conserved for all the other fish genes. Among the species compared, this feature also occurs in the Atlantic cod and in the zebrafish but with lesser frequency. Residues engaged in heme contact are always identical, which was interpreted as a naturally expected conservation among species of the amino acids linked to the enzyme active site (Murthy *et al.*, 1981).

From the set of organs tested, catalase mRNA was always present, with a marked predominance in liver and blood. In fact, hepatocytes are known to be plenty of peroxisomes, not only in mammals but also in other animals, including fishes and, in particular, brown trout (Lazarow, 1994; Rocha and Monteiro, 1999; Rocha *et al.*, 1999). Despite peroxisomes are the main catalase location (de Duve and Baudhuin, 1966), the enzyme also has a cytosolic location in specific cell types, such as the erythrocytes of many species (Maral *et al.*, 1977; Žikić *et al.*, 2001; Wilhelm Filho *et al.*, 2001). In this way, it is logical that catalase mRNA expression would be elevated in brown trout liver and blood.

Recently, a catalase expression pattern based on semi-quantitative RT-PCR was conducted on thicklip grey mullets (Bilbao *et al.*, 2006), with the adult male presenting higher expression in heart, liver, blood and muscle, and a low expression in spleen. The female pattern was somewhat different, with a very high expression in spleen and liver, and a low expression in muscle. Thus, we may speculate that gender differences may exist in brown trout organ expression of catalase, which can lead us to complement this study with female data in a future work.

Studies on seasonal catalase activity in aquatic organisms are scarce and involve mainly molluscs (Orbea *et al.*, 1999b; Nyiogi *et al.*, 2001) and, recently, a fish (Solé *et al.*, 2006) as bioindicators against marine or organic pollution. In brown trout, catalase activity variation in hepatic peroxisomes along the reproductive cycle has been already reported (Rocha *et al.*, 2001; 2004). Now we studied catalase expression variation in the same conditions. To our knowledge, this was

the first report on seasonal catalase gene expression in an animal species, and covering a full breeding cycle of an annual spawner.

In brown trout female liver, catalase expression levels were shown to be higher in postspawning and early vitellogenesis, significantly decreasing in late vitellogenesis and prespawning, along with the increasing estrogenic stimulus that accompanies the ovary maturation towards spawning. Although a positive correlation between catalase expression and its functionality/activity may not always be the case (Nakamura *et al.*, 1999; Sindhu *et al.*, 2005), the pattern we found for its expression in female brown trout liver agrees well with the described enzymatic activity variation in hepatic peroxisomes (Rocha *et al.*, 2001; 2004). Furthermore, this pattern is also consistent with the reduction in peroxisome individual and total volumes (Rocha *et al.*, 1999), and also urate oxidase and palmitoyl-CoA oxidase activities previously determined in female brown trout liver (Rocha *et al.*, 2001; 2004; Resende *et al.*, 2005). Finally, it shows a negative relation with the corresponding plasma testosterone and estradiol levels (Rocha *et al.*, 2001; 2004). Lowest levels of expression for females were reached in prespawning, which made this the only season when significant differences between genders were observed. No variation along the year was observed for catalase expression in males, which naturally lack estradiol regulation. These results support our earlier hyphotesis (Rocha *et al.*, 1999) about the estrogenic regulation of peroxisomes and peroxisomal enzymes.

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6. Kinetics of the Metabolic and Morphological Alterations in Brown Trout Hepatic Peroxisomes under Estradiol Influence

This chapter has the following co-authors:

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Summary

A regulatory interaction was suggested between estrogens and peroxisomes. We have previously reported significant changes on hepatic peroxisome morphological and biochemical parameters in female brown trout (*Salmo trutta* f. *fario*) along the reproductive cycle and also in immature fish under exogenous estradiol exposure. For a better understanding of the subjacent molecular mechanisms, we studied both biochemical and morphological aspects of the kinetics of estradiol exposure effects at 7, 15 and 30 days, as well as the recovery 15 days after hormone supply suspension. Peroxisomal enzyme activities were shown to be decreased after 7 days of estradiol treatment for catalase or 30 days for urate oxidase. Catalase further reduced its activity from day 15 to 30. Urate oxidase responded slowly to treatment suspension, being its activity still reduced 15 days after estradiol removal, while catalase recovery was already evident at that time. On the other hand, variations on peroxisomal morphological parameters were not so obvious. Only the relative peroxisome volumes within the whole hepatocyte and within its cytoplasm were significantly smaller at the end of the treatment, an effect that persisted after treatment suspension. The peroxisome mean volume in estradiol treated fishes tended to decrease with time, being smaller than in control fishes with a marginal significance only after estradiol withdrawal. An estrogen receptor inhibitor (ICI 182,780) was also included in an assay to test if the regulation of peroxisomes by estradiol is mediated by the estrogen receptor. On fish exposed simultaneously to estradiol and ICI, the activity of catalase was significantly higher than that observed on the group exposed to estradiol alone. However, the inhibitor ICI did not have a significant effect on urate oxidase activity nor on the peroxisomal morphological parameters. Taken together, these data suggest that the estrogen receptor might be involved in the mechanism. As to the kinetics, we conclude that some peroxisomal parameters (at least catalase activity) respond more rapidly to the presence and absence of estradiol, whereas other (such as urate oxidase activity and peroxisome size) respond much slower to estradiol, both in terms of induction and of recovery, probably due to a higher number of mechanistic levels between stimulus and observed effects.

Introduction

There is evidence that peroxisomes can be affected by estrogenic compounds. The uropygial glands of mallards are a remarkable witness of that relationship. An induction of peroxisome proliferation was observed in these glands in females during the mating season and in males upon estradiol treatment (Bohnet *et al.*, 1991). The peroxisomal enzymes that are involved in the synthesis of female sex pheromones in the uropygial glands were also induced by administration of estradiol to males, as well as to nonmating females (Bohnet *et al.*, 1991). In this gland, also alcohol dehydrogenase, another peroxisomal enzyme, was considerably influenced by estrogen administration, with a significant increase in both gene expression and enzyme activity (Hiremath *et al.*, 1992). Moreover, it was recently found that estradiol and several xenoestrogens are able to induce peroxisomal proliferation in zebrafish liver. This evaluation was based on increased values for peroxisomal surface and numerical densities, and the peroxisomal β-oxidation enzyme acyl-CoA oxidase activity (Ortiz-Zarragoitia and Cajaraville, 2005).

In brown trout (*Salmo trutta* f. *fario*), endogenous estradiol is at maximum levels in late vitellogenic and prespawning females (Fostier *et al.*, 1983; Rocha *et al.*, 2001; 2004b), coinciding with a significant reduction in hepatic peroxisome individual and total volumes, determined by stereological methods in transmission electron microscopy (Rocha *et al.*, 1999). Additionally, a biochemical approach to brown trout liver peroxisomes demonstrated a simultaneous decrease on the activities of catalase, palmitoyl-CoA oxidase and urate oxidase, the latter being almost null during late vitellogenesis (Rocha *et al.*, 2001; 2004b; Resende *et al.*, 2005). The effect of exogenous estrogens was also tested *in vivo*. The exposure of immature specimens to waterborne estradiol and alkylphenols, a class of xenoestrogens, for a period of 30 days led to similar trends, though to a much lesser degree in the case of the alkylphenols (Rocha *et al.*, 2004a).

From the above, estradiol treatment can apparently generate divergent results in different species and organs. This can be due to the different experimental methods employed or, otherwise, explained by species differences concerning physiology, biochemistry and sensitivity. In any case, the molecular mechanisms underlying these events are not completely clear and further studies are needed.

On the other hand, kinetics of the peroxisomal changes after exposure to estradiol is not yet available in any species.

To test the hypothesis that the interaction between estradiol and peroxisomes is directly mediated by a nuclear estrogen receptor, we exposed brown trout juveniles both to estradiol and to estradiol plus ICI 182,780, a known estrogen receptor inhibitor (Wakeling and Bowler, 1992; Kuiper *et al.*, 1998). In addition, we studied the kinetics of estradiol exposure during 30 days and the recovery within 15 days after estradiol supply suspension.

Materials and methods

Chemicals

17β-Estradiol, 3-(N-morpholino)-propanesulfonic acid (MOPS), bovine serum albumin (BSA), phenol, 4-amino-antipyrine, horseradish peroxidase, azide, cofactors and substrates for enzyme assays and 3,3'-diaminobenzidine (DAB) were obtained from Sigma-Aldrich (St. Louis, USA). ICI 182,780 was purchased from Tocris Bioscience (Ellisville, USA). Rainbow Trout Vitellogenin ELISA Kit was purchased from Biosense Laboratories (Bergen, Norway). All other chemicals used were of reagent grade and were from Merck (Darmstadt, Germany).

Animals and sampling

One-year-old farmed trout weighing 42 ± 19 g (mean ± standard deviation) were obtained from a governmental aquaculture facility. Before the beginning of the experiments, the animals were subjected to a 7 days period of aclimatation in tile coated raceway tanks. The 250 L of water in each tank were renewed every two days, being the water constantly aerated by diffusion bombs and mechanically filtrated. The water quality was controlled by the frequent measurements of several parameters: pH, gH (total hardness), dissolved oxygen and contents in ammonium, nitrates and nitrites. Animals were fed a commercial diet, following routine procedures. Three groups were established: a) control, b) estradiol and c) estradiol plus ICI. Estradiol and estradiol plus ICI were added to the water, in

closed circuit, after water replacement and cleaning of the tanks, which occurred 3 times a week. For both groups, estradiol was used in a nominal concentration of 50 µg/L of water. Because of its hydrophobic nature, the hormone was dissolved in 30 ml of a saline ethanol solution [absolute ethanol: NaCl 0.9% (1:1)] prior to adding to the water. The estradiol inhibitor, ICI, was added in a nominal concentration of 12.5 µg/L and prepared in the same way. In the control group, only an equal volume of saline ethanol solution was added to the water. To stop estradiol treatment, the animals were moved to a clean water tank treated in the same way as the control tank. At each sampling time – 7, 15, 30 and 45 days (15 days after treatment suspension) – 10 specimens of the control and of the estradiol groups were collected by random net fishing. Additionally, at 30th day, 10 animals from the estradiol plus ICI group were collected. Before manipulation, fishes were deeply anaesthetised for a few minutes by immersion in 1 ml/L aqueous solution of ethylene glycol monophenyl ether, then weighed and measured in length. Livers were then quickly removed from fishes chilled on ice, and retrogradely perfused at 4º C with a heparinized (5 IU/ml) isosmotic buffer for salmonids (Rocha *et al.*, 2003) at a physiological flow rate of approximately 5 ml/min/Kg of body weight, via the hepatic vein(s). Room temperature was 18º to 20º C. After removal, livers were immediately weighed for hepatosomatic index determination and cut into slices according to Rocha and coworkers (1999) in order to assure equal sampling probabilities for all liver fragments, gathered and kept for further use in different tasks and purposes. Gonads were also removed and weighed for gonadosomatic index estimation.

Plasma collection and preparation of tissue homogenates

Blood samples were taken by caudal puncture with heparinized syringes and immediately centrifuged in heparinized tubes at 1,500 g for 10 min, at 4º C. The plasma was then removed and stored at -80º C with 1% of a diluted solution of phenylmethylsulfonylfluoride (PMSF) until analysis. The PMSF saturated solution in absolute ethanol was pre-prepared and diluted 10x in the same solvent.

A sample of each liver was homogenized in chilled homogenization buffer with pH 7.4 [250 mM sucrose, 5 mM MOPS, 1 mM EDTA (ethylenediaminetetraacetic acid), 0.1% ethanol saturated with PMSF and Triton X-100 0.5%] (Goldenberg, 1977), using a Potter-Elvejhem homogenizer at 1,000 rpm, held at 4º C. The

homogenized was filtered through a 95 µm mesh net, then the volume was adjusted to 10 ml/g of liver with the same buffer and centrifuged at 10,000 g for 10 min. The supernatant was collected and aliquots were stored at -80º C until further processing.

Plasma vitellogenin levels determination

For control of basal estradiol effects, vitellogenin, a biomarker of estradiol exposure, was measured in both treated and untreated fishes. Plasma vitellogenin levels were determined by enzyme-linked immunosorbent assay (ELISA) using a commercially available rainbow trout vitellogenin ELISA kit (Biosense, Bergen, Norway), according to the manufacturer's instructions. All the assays were performed in duplicate, with different dilutions of each sample.

Biochemistry

All assays were run in a spectrophotometer connected to a circulating water system for temperature regulation in the cuvette compartment. Total protein content was determined according to Lowry and coworkers (1951), using BSA as standard, and results were expressed in BSA equivalents. Enzymatic assays were performed in duplicate, to calculate a mean value and to assure results reproducibility. Different sample dilutions were used in order to obtain linearity of enzymatic activities in time and proportionality in protein amount.

Catalase activity: The assays for catalase activity were carried out according to the method described by Aebi (1984), as reported by Rocha and coworkers (2003). The reaction mixture consisted of 10 mM hydrogen peroxide (H_2O_2) in 50 mM sodium phosphate buffer, pH 7.0. To start the enzymatic reaction, 50 µl of 33.3 fold diluted sample were added to 2,950 µl of medium (2,000 fold final dilution), at 20º C. For a duplicate, 50 µl of 66.7 fold diluted sample were also added to 2,950 µl of medium (4,000 fold final dilution), at 20º C. Consumption of H_2O_2 was measured by the values of absorbance (OD) at 240 nm at initial time and after 30 seconds of reaction. The enzymatic activity was given by the first order rate constant, K, for the degradation of H_2O_2 (Aebi, 1984): $K = \ln (ODt_0/ODt_{30})/30$.

After application of the dilution factor, it was ultimately expressed in s^{-1} per mg of protein.

Urate oxidase activity: Measurement of urate oxidase activity was based on the production of H_2O_2 and followed the procedure of Cablé and coworkers (1993) with minor modifications (Rocha *et al*., 2003). Reaction mixture contained 1 mM uric acid, 1 mM phenol, 0.082 mM 4-amino-antipyrine, 2 IU/ml horseradish peroxidase, 0.06% BSA, 10 mM azide and 10 µM FAD in 50 mM potassium phosphate buffer, pH 8.0. Azide was included to avoid the interference of catalase, according to Leupold and coworkers (1985). The enzymatic reaction was started by the addition of 25 µl of a properly diluted sample (2, 4, 8 or 16 fold) to 650 µl of reaction mixture at 25° C and, subsequently, the absorbance increase at 500 nm (ΔOD) was measured for 10 min. To abolish the effect of non-specific reactions, a baseline curve made with medium without sample was subtracted from the absorbance increase in each assay. The amount of H_2O_2 produced was calculated from the equation of a calibration curve previously obtained (Rocha *et al*., 2003): $[H_2O_2] = 185.07 \times \Delta OD$. Enzymatic activity was given by the application of the dilution factor to this value and ultimately expressed in $nmolmin^{-1}$ per mg of protein.

Cytochemistry

For the detection of catalase activity at transmission electron microscopy level, very small pieces of tissue (sliced under a stereomicroscope) were fixed in 2.5% glutaraldehyde buffered with 0.1 M sodium phosphate pH 7.4, at 4° C for 2 h. After several rinses in phosphate buffer and Tris-HCl buffer 0.1 M pH 8.5, the pieces were incubated for 2 h in medium containing 0.12% H_2O_2 and 2 mg/ml of DAB in Tris-HCl buffer (Veenhuis and Wendelaar Bonga, 1979) and then rinsed again with Tris-HCl buffer and phosphate buffer. The fragments were postfixed in 1% osmium tetroxide in phosphate buffer with 1.5% potassium ferrocyanide, for 2 h at room temperature. After dehydration in ethanol, the fragments were embedded in Epon. Ultrathin sections were observed without further staining in a JEOL 100CXII.

Stereological Methodology

Estimatives of relative and absolute peroxisome morphological parameters in the different groups of animals were obtained at electron microscopy level, using catalase cytochemistry for a correct identification of the organelle. From each animal, 5 tissue blocks were cut and a sole grid was made from each one. Then, an average of 8 fields was systematically photographed from each grid, disregarding areas not occupied by hepatocytes. The whole 1,800 photographs of this study were taken at 5,300x of magnitude, with a final printing magnification of 15,900x for the stereological analysis. The morphological parameters analysed were the relative peroxisome number [N_v(Peroxisome, Hepatocyte)] and volume [V_v(Peroxisome, Hepatocyte)] within the whole hepatocyte, the relative peroxisome volume within the cytoplasm [V_v(Peroxisome, Cytoplasm)], and the absolute mean volume of a peroxisome [$\bar{v}$(Peroxisome)]. They were determined using the methods detailed and validated by Rocha and coworkers (1999).

Statistics

Data are reported as means per group of animals, with the respective standard deviations (SD). After testing the normality and the homogeneity of variances, a two-way ANOVA was performed to detect the effects of estradiol and estradiol plus ICI on peroxisomal enzyme activities, peroxisome morphological parameters, hepatosomatic index and plasma vitellogenin levels. In order to achieve normalization and homogenization of variances in the analysis of enzymatic activities, square root transformation was made when appropriate. After a significant ANOVA, Tukey and Newman-Keuls post-hoc tests for multiple comparisons between means were further applied. Differences were considered significant when $p \leq 0.05$ for both tests. When differences were significant according to Newman-Keuls test but not according to Tukey test, they were considered marginally significant ($p \cong 0.05$).

Results

Enzymatic activities

Under two-way ANOVA, significant group and time effects were detected for both enzyme activities tested ($p \leq 0.001$). The interaction between group and time was also significant, with $p \leq 0.05$ for catalase activity and $p \leq 0.001$ for urate oxidase activity. In control fishes, none of the enzymes showed activity variation with time.

Comparatively to control groups, catalase activity in estradiol treated fishes was shown to be drastically reduced just after 7 days of hormone exposure, reaching the lowest level at day 30 and recovering to day 7 level within 15 days after treatment suspension (Fig. 1). It was significantly lower than that of untreated fishes in every sampling time. Animals exposed to estradiol plus ICI showed a catalase activity that was significantly higher than that from animals exposed to estradiol alone, and significantly lower than that of the control group.

Urate oxidase activity significantly declined from exposure day 7 to 30 and was sustained 15 days following estradiol supply suspension (Fig. 1). The enzyme activity observed in estradiol treated animals was significantly lower than that of untreated ones only at day 30 and also after treatment suspension. In the case of urate oxidase, the estrogen receptor inhibitor ICI did not show any effect.

Stereology of peroxisomes

Significant differences ($p \leq 0.05$) for peroxisomal absolute and relative parameters were detected by two-way ANOVA. Time effect was only observed in N_v(Peroxisome, Hepatocyte) and $\bar{v}$(Peroxisome). The group effect was noted in V_v(Peroxisome, Hepatocyte), V_v(Peroxisome, Cytoplasm) and $\bar{v}$(Peroxisome). Interaction between time and group was not significant in any case. Neither morphological parameter showed activity variation with time in control fishes.

Comparatively to the controls, N_v(Peroxisome, Hepatocyte) in estradiol treated fishes of the same timepoints was not significantly affected. After treatment suspension, the parameter was slightly elevated, becoming significantly higher

than at day 7 of estradiol exposure (Figs. 2 and 3). Animals exposed to estradiol plus ICI did not differ from the other groups (Figs. 2 and 4).

Despite $\bar{v}$(Peroxisome) was not significantly different between control and estradiol treated animals until day 30, the mean values continuously tended to decrease under hormonal influence, an effect that remained after treatment suspension. This decrease caused a marginally significant difference between estradiol treated fishes and controls at day 45 (Fig. 2) and a significant difference between day 7 and day 45 of estradiol exposure (Figs. 2 and 3). Fishes treated with estradiol plus ICI showed the same $\bar{v}$(Peroxisome) as fishes treated with estradiol alone. However, this value is significantly lower than the value found in controls (Figs. 2 and 4).

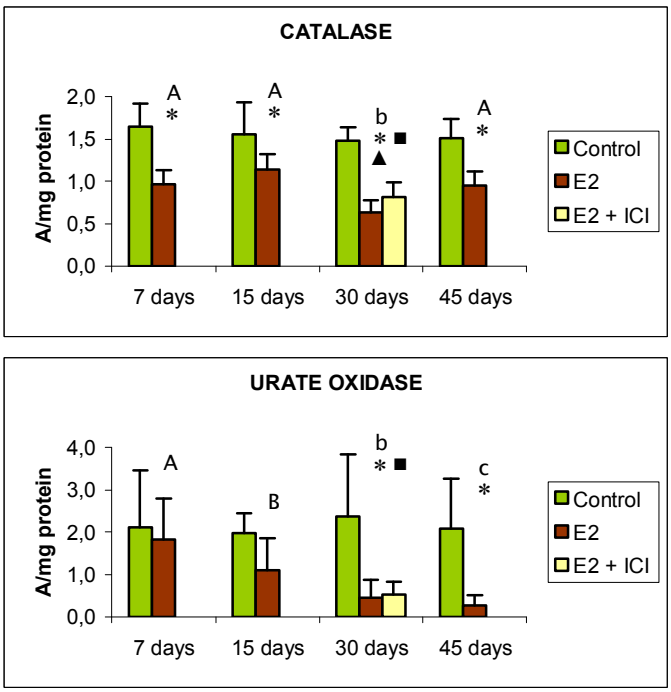

Fig. 1 – Activity (A) of hepatic peroxisomal enzymes, expressed as s^{-1} (catalase) or $nmol.min^{-1}$ (urate oxidase), in estradiol (E2) and estradiol plus ICI (E2 + ICI) exposed fishes against control groups. Estradiol treatment was suspended at day 30. Values are reported as mean + SD. Differences among sampling times within estradiol treated groups are considered significant ($p \leq 0.05$) when different letters and different case types are simultaneously used. Asterisks represent significant differences ($p \leq 0.01$) between control and estradiol treated fishes. Squares represent significant differences ($p \leq 0.05$) between control and estradiol plus ICI treated fishes. Triangles represent significant differences ($p \leq 0.05$) between estradiol and estradiol plus ICI treated fishes.

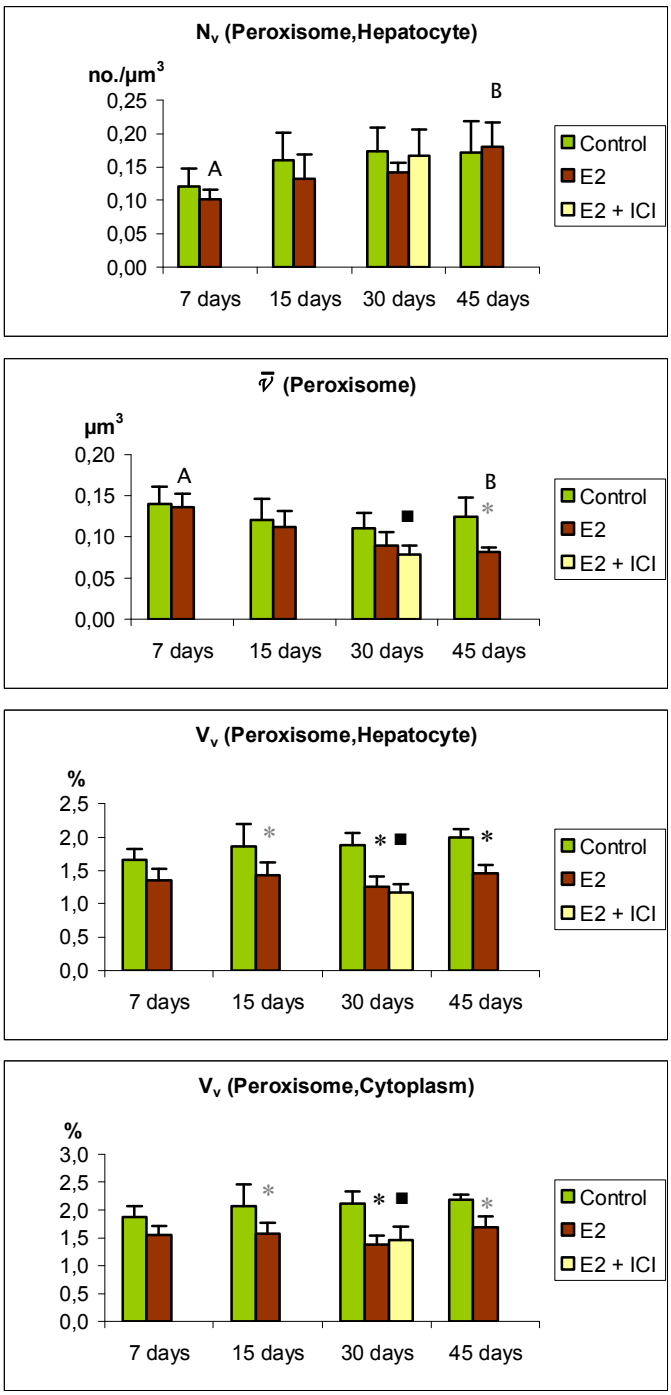

Fig. 2 – Absolute and relative parameters in liver peroxisomes of estradiol (E2) and estradiol plus ICI (E2 + ICI) exposed fishes against control groups. Estradiol was suspended at day 30. Values are reported as mean + SD. Different upper cases represent significant differences ($p \leq 0.05$) among sampling times within estradiol treated groups. Asterisks and squares represent significant differences ($p \leq 0.05$) between control and estradiol treated fishes, and between control and estradiol plus ICI treated fishes, respectively (grey asterisks represent marginal differences ($p \cong 0.05$). N_v(Peroxisome, Hepatocyte): relative peroxisome number within the whole hepatocyte; $\bar{v}$ (Peroxisome): absolute mean volume of a peroxisome; V_v(Peroxisome, Hepatocyte): relative peroxisome volume within the whole hepatocyte; V_v(Peroxisome, Cytoplasm): relative peroxisome volume within the cytoplasm.

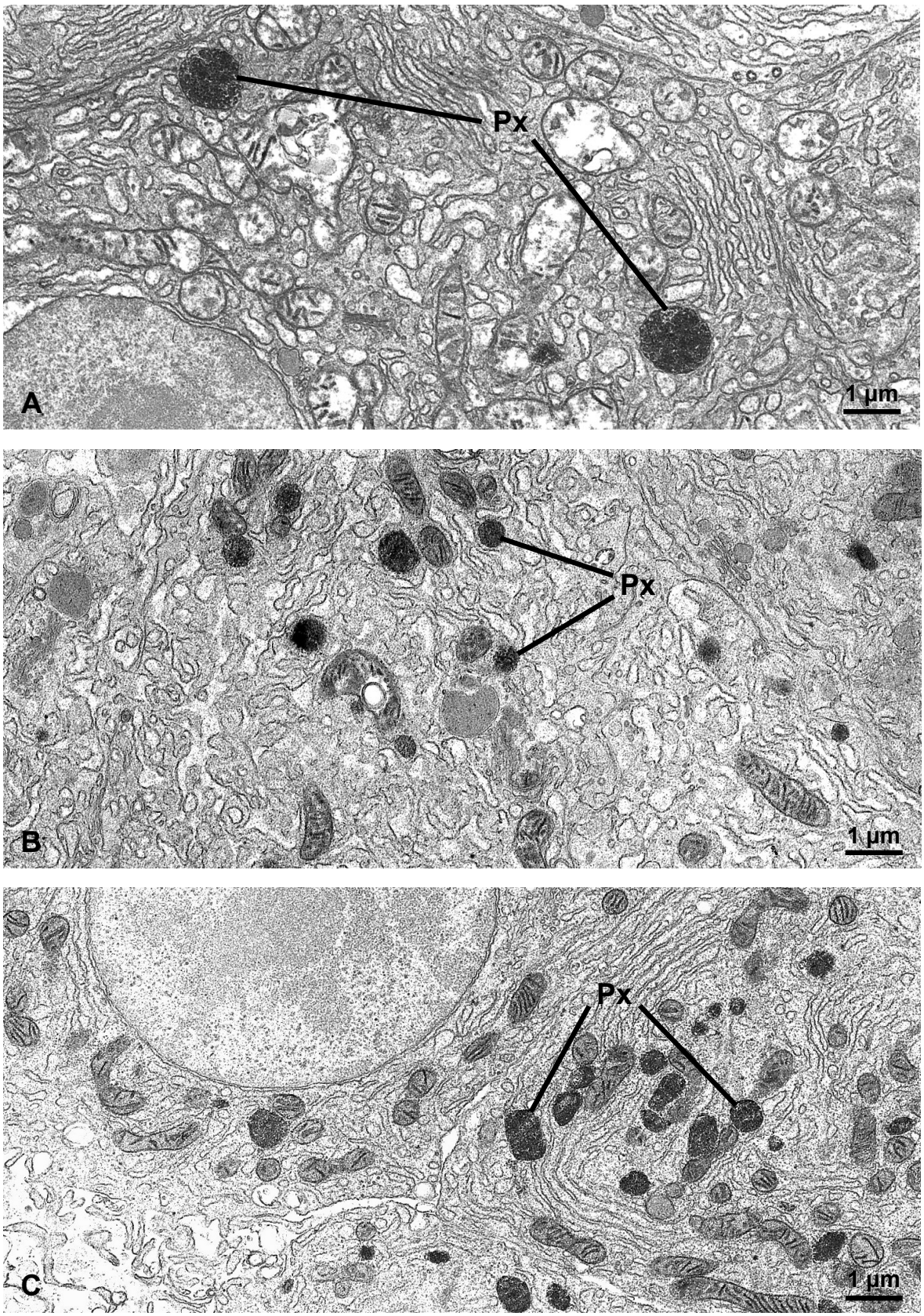

Fig. 3 – Ultrathin sections of brown trout hepatocytes under estradiol effect, with peroxisomes (Px) stained after DAB reaction for the detection of catalase. A: day 7; B: day 30; C: day 45 (day 15 after treatment suspension).

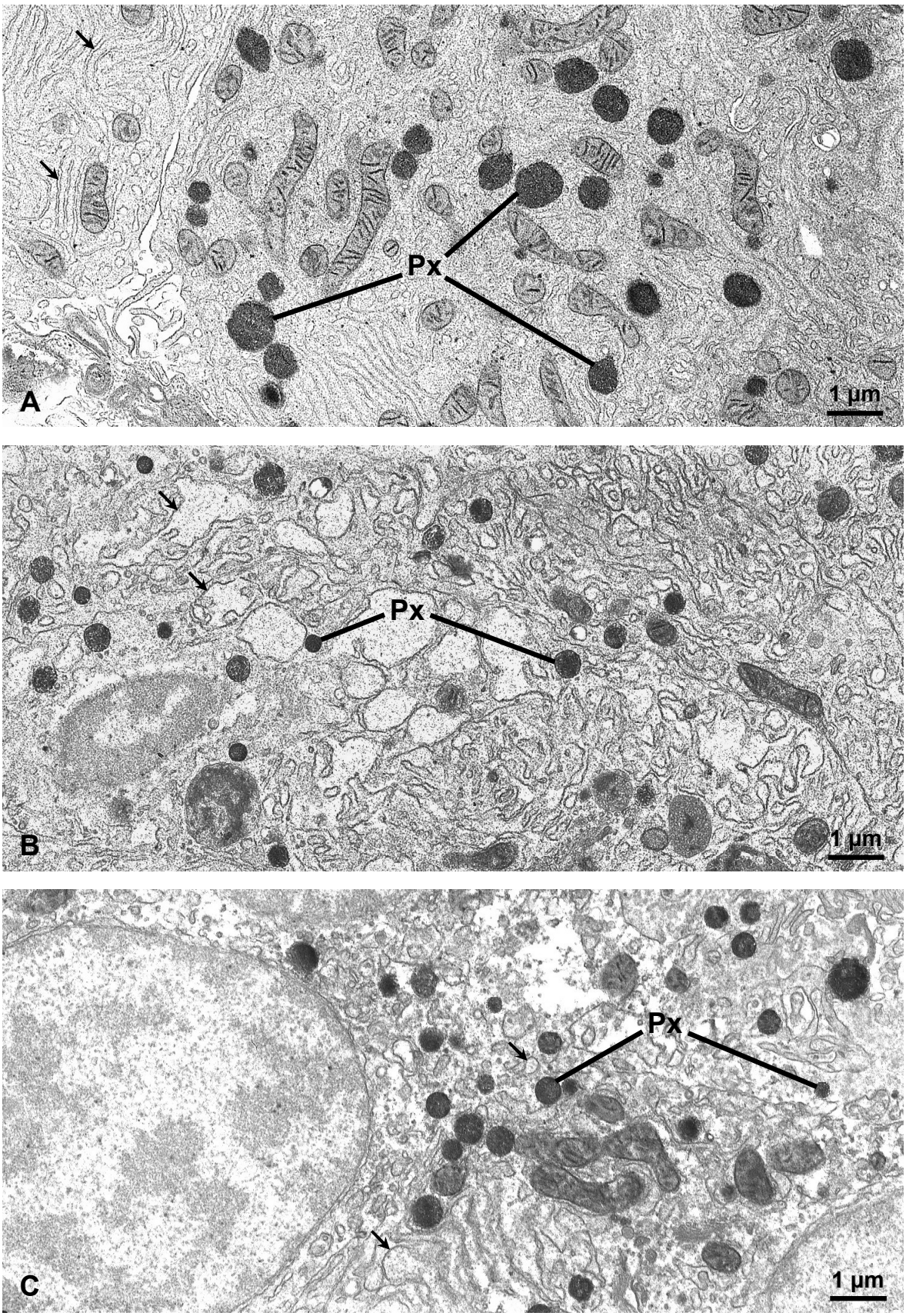

Fig. 4 – Ultrathin sections of brown trout hepatocytes taken at day 30, with peroxisomes (Px) stained after DAB reaction for the detection of catalase. Arrows: endoplasmic reticulum cisternae. A: control; B: estradiol; C: estradiol plus ICI.

Fishes treated with estradiol showed a constant V_v(Peroxisome, Hepatocyte) during the period of exposure (Fig. 2). At days 15 and 30, this value was significantly lower than V_v(Peroxisome, Hepatocyte) of untreated fishes, with a marginal difference at day 15. The significant difference between estradiol treated and untreated fishes observed at day 30 persisted after hormone supply suspension. Under the simultaneous presence of estradiol and the estrogen receptor inhibitor, this parameter showed similar values as seen in the group exposed to estradiol alone (Fig. 2).

Similarly, V_v(Peroxisome, Cytoplasm) of treated groups remained constant in time and significantly lower than V_v(Peroxisome, Cytoplasm) of control groups at days 15 and 30, with a marginal difference at day 15 (Fig. 2). After cessation of the estrogenic stimulus, this stereological parameter reapproached the non-stimulated group level, with a marginal difference. Once again, estradiol plus ICI did not show a significant effect in this peroxisomal parameter, when compared to the effect of estradiol alone (Fig. 2).

An evidence of estradiol exposure was the clear enlargement of the endoplasmic reticulum cisternae. These appeared much dilated at day 30 than at day 7 (Figs. 3A and 3B), and also in hormone treated fishes than in control fishes at day 30 (Figs. 4A and 4B).

Hepatosomatic index and plasma vitellogenin determination

Under two-way ANOVA, significant group and time effects were detected for hepatosomatic index ($p \leq 0.01$) and plasma vitellogenin levels ($p \leq 0.001$). The interaction between group and time was also significant ($p \leq 0.001$).

In relation to control groups, waterborne estradiol exposure caused a significant increase of the hepatosomatic index, noticeable as early as day 7 and remaining above control levels until day 30 (Fig. 5). At this time, it was also significantly higher than at day 7. After suspension of hormone supply, this value came to control levels, becoming significantly lower than the values of days 15 and 30. The hepatosomatic index of fishes treated with estradiol plus ICI was significantly higher than the value for controls but also significantly lower than the value for fishes treated with estradiol alone.

Plasma vitellogenin level was significantly higher in estradiol treated fishes than in untreated fishes just after 7 days of hormone exposure, reaching the highest level at day 30 (Fig. 5). After treatment suspension, it decreased to the level of day 15. Animals exposed to estradiol plus ICI showed a vitellogenin level significantly lower than the level of animals exposed to estradiol alone, and also significantly higher than that of the control group.

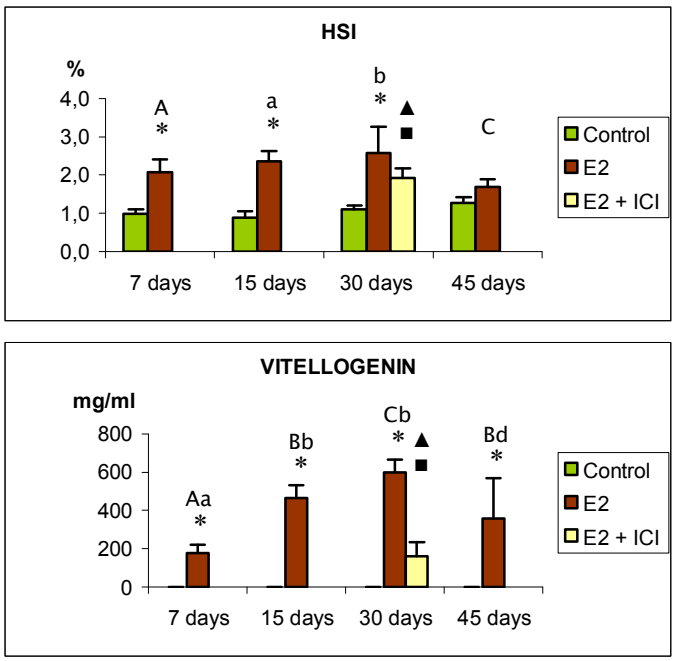

Fig. 5 – Hepatosomatic index (HSI) and plasma vitellogenin values for estradiol (E2) and estradiol plus ICI (E2 + ICI) exposed fishes against control group. Estradiol was suspended at day 30. Values are reported as mean + SD. Asterisks and squares represent significant differences ($p \leq 0.01$) between control and estradiol treated fishes, and between control and estradiol plus ICI treated fishes, respectively. Triangles represent significant differences ($p \leq 0.01$) between estradiol and estradiol plus ICI treated fishes. For hepatosomatic index: Differences among sampling times within estradiol treated groups are considered significant ($p \leq 0.01$) when different letters and different case types are simultaneously used. For vitellogenin: Differences among sampling times within estradiol treated groups are considered significant ($p \leq 0.05$) when they don't have any of the strictly same letters (the same case and letter simultaneously).

Discussion

In face of the evidence indicating that brown trout hepatic peroxisomes are affected by endogenous and exogenous estrogens (Rocha *et al.*, 1999; 2001; 2004a; 2004b; Resende *et al.*, 2005), we now conducted a study to know more about the kinetics of the alterations observed on some peroxisomal morphofunctional parameters under exogenous estradiol exposure and recovery, as well as the possible role of the nuclear estrogen receptor in the mechanism.

The response of catalase and urate oxidase in the liver of brown trout juveniles after 30 days of exposure to waterborne estradiol was in agreement with the described enzyme activities of mature females along the reproductive cycle (Rocha *et al.*, 2001; 2004b; Resende *et al.*, 2005). Late vitellogenesis and prespawning, which correspond to maximum endogenous estradiol concentrations (Fostier *et al.*, 1983; Rocha *et al.*, 2001; 2004b), were the seasons with the lowest activity levels for these enzymes. Catalase expression level was, as well, reported to be lowest in these seasons, as stated in the preceding chapter. The kinetics of the enzymatic responses to the hormone, however, was different for each enzyme in our experiment. Catalase response was faster, with a significant activity reduction in relation to controls detected just at exposure day 7. It further decreased until day 30 and promptly recovered to day 7 levels within 15 days after exposure suspension. Urate oxidase showed a slower response. Although continuously decreasing, a significant difference from controls in its activity was only detected after 30 days of exposure and this effect persisted 15 days following treatment suspension. The known estrogen receptor inhibitor ICI also evoked different effects on the activity of each enzyme. It showed a partial but significant restrainer effect of estradiol on catalase but not on urate oxidase. The partial inhibitory action of ICI on estradiol may be explained by the fact that the dosage of inhibitor used in these assays (ICI:estradiol ratio of 1:9 in molarity or 1:4 in mass) was much below described dosages that completely inhibited the effects of the hormone. Molarity ratios of 3:1 (Gore, 2002) and 10:1 (Gingerich and Krukoff, 2005) were used to effectively inhibit estrogenic effects. Anyway, as the most effective dosages of ICI (or of ICI:estradiol ratios) in animal models is most basically unknown, including in our trout model, we decided on a conservative approach that would provide us hints about a direct connection of the observable effects in peroxisomes with an estradiol mediated effect. In this

way, the reason for an apparent null effect of ICI on the action of estradiol on urate oxidase might just be a matter of dosage.

The important at this point is that our data support that at least some of the changes observed in peroxisomes are directly dependent on estrogen receptor activation after estradiol exposure. Nevertheless, parallel processes, such as action of estradiol metabolites and involvement of other receptors, can not be discarded. In addition, the data support that, under these circumstances, catalase activity is more rapidly affected by this hormone than other peroxisomal enzymes, such as urate oxidase, being the regulatory effect probably done via gene expression tuning, although urate oxidase activity responses to other estrogen stimuli was shown to be more intense (Rocha *et al.*, 2001; 2004a; 2004b; Resende *et al.*, 2005).

Although previous data on brown trout juveniles pointed to considerable changes in peroxisomal morphological parameters with estradiol exposure in a 39-day estradiol exposure trial (Rocha *et al.*, 2004a), the present results were not so conspicuous. The most relevant variations herein were V_v(Peroxisome, Hepatocyte) and V_v(Peroxisome, Cytoplasm). Neither N_v(Peroxisome, Hepatocyte) nor $\bar{v}$(Peroxisome) showed significant alterations during the exposure time, although a trend for a time increase of N_v(Peroxisome, Hepatocyte) and decrease of $\bar{v}$(Peroxisome) was noted. In parallel with this, the hepatosomatic index was significantly increased during the whole exposure time. It is known that hepatomegaly may be caused both by cell hyperplasia (Korsgaard *et al.*, 1986) and/or by hypertrophy (Emmersen *et al.*, 1979; Hornstein *et al.*, 1992; Sehgal and Goswami, 2001; Olivereau and Olivereau, 2004). Rocha (2000) showed that in brown trout hepatocytes mitosis seems to be the main mechanism by which the liver gains weight under estradiol influence in the natural breeding cycle. Cell volume remains fairly constant or tends to decrease in vitellogenesis. Since N_v(Peroxisome, Hepatocyte) did not change under waterborne estradiol exposure, the observed reduction in peroxisomal relative volumes must be due to a real decrease in $\bar{v}$(Peroxisome), although only a marginally significant difference was detected in this parameter.

Interestingly, the tendencies observed in N_v(Peroxisome, Hepatocyte) and $\bar{v}$(Peroxisome) continued after estradiol withdrawal and were reflected 15 days after treatment suspension. At this time, the differences from day 7 of hormone exposure became significant. In the conditions in which this study was

undertaken, the effect of waterborne estradiol exposure on hepatic peroxisomes morphology was gradual in time and observed even after exposure end, contrasting with its effect on the activity of enzymes like catalase. Urate oxidase response was between these.

Not naturally present in males or immature fish, the estrogen-inducible protein vitellogenin has been used as an indicator of estrogenic compounds exposure (Kime, 1999; Tyler *et al.*, 1999; Hiramatsu *et al.*, 2006). In the same way, the hepatosomatic index determination was integrated in a screening assay for these chemicals in juvenile trouts (Thorpe *et al.*, 2000). To overthrow the hypothesis of estradiol degradation in the water system used, plasma vitellogenin levels and hepatosomatic index measurements were made on both treated and untreated animals. The elevated values determined for both parameters in hormone treated groups comparatively to controls revealed an effective hormone exposure. Moreover, 15 days after treatment suspension, plasma vitellogenin levels were still raised in the estradiol treated group (with the exception of one animal, which caused a major standard deviation for this value). A study of plasma vitellogenin levels and its hepatic expression made in male sheephead minnow showed that, whereas hepatic vitellogenin mRNA quickly decreases after estrogen exposure cessation, plasma vitellogenin clearance is dose- and time-dependent, being the protein detectable in the plasma at considerable levels for several months thereafter (Hemmer *et al.*, 2002). Estradiol effective exposure was further confirmed at electron microscope level by the characteristic enlargement of the endoplasmic reticulum cisternae, commonly associated with vitellogenin mass production after estrogen induction (Peute *et al.*, 1985; Bieberstein *et al.*, 1999).

One of the purposes of this study was to clarify if activation of estrogen receptor was really required for the peroxisomal events to occur in the estradiol exposure assay. In theory, estrogen receptor-independent mechanisms may account for the peroxisomal changes, as reported for several phenomena influenced by natural and synthetic estrogens (Dubey *et al.*, 2001; Budhiraja *et al.*, 2003; Lee *et al.*, 2007). ICI showed an estradiol inhibitory action on catalase activity but not on urate oxidase activity neither on peroxisomal parameters. Possibly, the inhibitory effect of ICI was not perceptible in these cases due to a slower or less intense response to the estrogenic stimulus. Although ICI is a proven estrogen receptor inhibitor (Wakeling and Bowler, 1992; Kuiper *et al.*, 1998), it was also reported to show, in particular circumstances, a tissue-specific behaviour in sea bream, *Sparus aurata*, either acting as an estradiol agonist in liver or having no

noticeable effect in testis (Pinto *et al*., 2006). Furthermore, ICI behaviour in sea bream liver was variable, depending whether it was simultaneously or priorly administered to estradiol and its effect on the expression of several genes regulated by the hormone was also variable: apparently, ICI pretreatment synergistically potenciated estradiol effect on the estrogen receptor ERβb gene expression but inhibited its effect on the estrogen receptor ERα gene expression (Pinto *et al*., 2006). Regardless of this, our data do suggest that changes we saw in catalase activity under estradiol exposure seem to require estrogen receptor activation, independently of potential receptor-independent actions. By the other hand, the fact that ICI did not influence urate oxidase down-regulation exerted by estradiol may suggest an estrogen receptor-independent regulatory mechanism, but a direct estrogen receptor-dependent shutdown mechanism can not be ruled out because we tested a relatively low nominal ICI:estradiol ratio. Anyway, the fact that urate oxidase remained repressed after estradiol withdrawal may lead us to hypothesize that estradiol regulation may involve, indeed, estrogen receptor-independent actions and/or multicascade events. In fact, one must bear in mind that estradiol and estrogen receptor signalling occur in multifaceted mechanisms, including estradiol cell-surface signalling, a nongenomic mechanism (Hall *et al*., 2001).

In summary, we conclude that the estrogen receptor is most likely involved in the estradiol regulation mechanism of peroxisomes, however not excluding that additional estrogen receptor-independent mechanisms may occur. We further conclude that the regulatory mechanism for each peroxisomal feature has specific kinetics, with catalase being more sensitive to the hormone and, thus, more hastily influenced by its presence or absence than urate oxidase and peroxisome size. Behind these differences may be the existence of a variety of multicascade occurrences between stimulus and observed effects.

Acknowledgments

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7. CONCLUDING REMARKS

Concluding Remarks

This thesis focused on relations between the breeding cycle, estrogenic stimulus, PPARs and liver peroxisomes of brown trout. The work provided new data, supporting the suspected involvement of the estrogen receptor and PPARs in peroxisome regulation. New additional hypothesis on both aspects were derived.

1. Novel genes for brown trout were cloned and identified as PPARα, PPARβ, PPARγ and catalase, with the partially deduced amino acid sequences sharing high levels of homology with the corresponding peptides of other fish species.

2. PPARα mRNA predominated in white muscle, heart and liver. In male liver, its expression along the reproductive cycle was invariable, whereas in female liver it was much more expressed during early vitellogenesis and less expressed during late vitellogenesis than in the other seasons. PPARα mRNA level was higher for females than for males in early vitellogenesis. The female annual PPARα expression pattern was negatively related with its annual plasma estradiol levels, and with peroxisome morphology and enzyme activities. Taken together, the results suggest a role for PPARα in the regulation of the metabolic demands of trout cardiomyocytes and white muscle fibres, along with energy related functions in hepatocytes. The data also support the hypothesis that PPARα is under estradiol modulation, which apparently acts as a down-regulator.

3. Confirming the ubiquity and constitutive expression of PPARβ, its mRNA was found in all organs tested (heart, liver, head kidney, trunk kidney, spleen, testis, blood and white muscle), with the strongest overall expression among the three isotypes. It was particularly abundant in testis, heart, liver and white muscle. The seasonal hepatic PPARβ expression study revealed no significant variation along the female breeding cycle, contrasting with the male pattern, which showed a much higher expression in prespawning than in the other seasons, suggesting a positive connection with the hormonal events that are known to occur at the time, namely the abrupt rise of 11-ketotestosterone. This result might indicate the existence of specific functions for this PPAR isotype in male fishes.

4. With a much weaker global expression, PPARγ mRNA was only detected in trunk kidney and liver (by real-time RT-PCR) and also in spleen (by semi-quantitative RT-PCR). In female liver, PPARγ was more expressed in postspawning than in late vitellogenesis and prespawning, with early vitellogenesis showing an intermediate expression. As to males, its hepatic expression was higher in postspawning than in all the other seasons. These findings are compatible with the assignment of immune related functions for PPARγ in teleosts, herein in the brown trout probably in connection with liver remodelling via macrophagic activity occurring after spawning.

5. Catalase mRNA transcript was shown to be more abundant in liver and blood, followed by testis, white muscle and trunk kidney. Whereas hepatic catalase seasonal expression was constant in males, its expression in females was shown to be higher in postspawning and early vitellogenesis than in late vitellogenesis and prespawning. Differences between genders were detected in prespawning, with a higher level of expression for males. A positive correlativity was found between seasonal variation of catalase gene expression and its enzymatic activity in female liver. Additionally, this pattern showed positive and negative correlativities with peroxisome total volume and plasma estradiol levels variations, respectively. These results strengthen the hypothesis that there is an estrogenic regulation of peroxisomes and peroxisomal enzymes, with estradiol promoting a volume reduction of peroxisomes in hepatocytes, in parallel with a decrease of both gene expression and enzyme activity of catalase and, probably, other peroxisomal enzymes. Naturally, the conclusions can not be extrapolated to all the enzyme array of peroxisomes, but the data seem sufficient to extend our main hypothesis about peroxisome regulation by estradiol to a molecular level.

6. The supply of exogenous estradiol in a concentration of 50 µg/L of water to brown trout juveniles for a month produced basically the same effects on hepatic catalase and urate oxidase activities than the endogenous hormonal raise occurring in late vitellogenic and prespawning mature females. This assay reproduced with minor differences, attributable to inter-assay and inter-animal variability, a previous similar 30-day assay, giving additional information about the kinetics of the event. Although both enzymes were negatively influenced by the hormone, they exhibited distinct kinetics:

catalase responded promptly to waterborne estradiol, as well as to its withdrawal, whereas urate oxidase displayed a slower response in both cases. A relatively low concentration (12.5 µg/L of water) of a potent estrogen receptor inhibitor (ICI 182,780) affected estradiol action on catalase but not on urate oxidase.

7. The effect of waterborne estradiol exposure on hepatic peroxisomes morphology was less striking and somehow delayed when compared with catalase activity changes. Only the relative peroxisome volumes, both within the whole hepatocyte and within its cytoplasm, were reduced at the end of the treatment, an effect that persisted after treatment suspension. Peroxisome mean volume tended to decrease under estradiol exposure, but only reaching statistical significance after 45 days, meaning that the estrogenic stimulus takes some time (at least about 30 days under the assayed conditions) to induce a size decrease, which is maintained for at least 15 days after direct estrogen stimuli cessation. The estrogen receptor inhibitor ICI administered in a low dosage simultaneously with estradiol did not have a significant effect on these parameters. However, facing the results for catalase, a direct or indirect role of estrogen receptor activation regarding the gradual morphological changes in peroxisomes under estradiol stimulus can not be ruled out at this point.

Overall, in our trout model, PPARs expression vary among organs, between genders, and during the seasonal breeding cycle, with PPARα showing in females a seasonal profile that is compatible with a regulation by sex steroids, namely by estradiol. When plasma levels of the latter are high, in parallel with ovary maturation, expression of PPARα lowers. The results are in accordance with the known seasonal decrease of the peroxisomal PPARα-regulated acyl-CoA oxidase activity (Rocha *et al.*, 2001; 2004). Moreover, these changes may well be connected with the observed reduction of relative and absolute volumes of liver peroxisomes under estradiol presence, and also with the decrease in gene expression and enzyme activity of catalase, as well as in the activity of other enzymes. The data support that at least some of the changes seen in peroxisomes are dependent on estrogen receptor activation. Nevertheless, it should not be discarded that an estrogen receptor-independent action exerted by estradiol may also occur, as reported in other studies (Levin, 2005; Guo *et al.*,

2006). Finally, it can be further accepted that cross-talk between estrogen receptor and PPARα could exist in brown trout hepatocytes.

The proposed mechanism for peroxisomal estradiol regulation in brown trout hepatocytes (Fig. 1) is a hypothetical integration of our findings in brown trout with bi-directional cross-talk interactions known to occur in other models. Upon activation by the respective ligand, each nuclear receptor recognizes and binds to a specific nucleotide sequence in the promoter region of the gene under its regulation, subsequently activating the corresponding gene transcription (Fig.1 A-B and C-D) (Sørensen *et al*., 1998). Despite this, it was demonstrated *in vitro*, in construct models, that both PPARγ and PPARα could effectively regulate estrogen receptor target gene expression (Fig.1 F) (Keller *et al*., 1995; Lemberger *et al*., 1996). Likewise, it was also proven that the estrogen receptor was able to repress PPARγ mediated transcriptional activity in breast cancer cells (Fig.1 E) (Wang and Kilgore, 2002; Bonofiglio *et al*., 2005). This cross-talk mechanism between PPARs and estrogen receptors is possible due to a close resemblance between the response elements sequences recognized by each nuclear receptor. The estrogen response element (ERE) is a palindromic or inverted repeat of a short motif separated by three nucleotides – GGTCAnnnACTGG - (Klein-Hitpass *et al*., 1988), whereas the peroxisome proliferator response element (PPRE) is a direct repeat of a short motif separated by one nucleotide - AGGTCAnAGGTCA – (Tugwood *et al*., 1992). Although under physiological cellular conditions the DNA binding sequence of each receptor has more affinity for the corresponding response element, it is likely that competitive cross DNA binding will occur in the case of a greatly imbalanced concentration between these receptors, after activation by the respective ligands (Lemberger *et al*., 1996).

Our results in brown trout hepatocytes support the hypothesis that estradiol exerts an estrogen receptor-dependent down-regulation on PPARα gene expression (Fig. 1 G), not disregarding that estradiol regulation may also involve estrogen receptor-independent actions. The elevated estradiol levels observed in late vitellogenic females additionally allows generation of such a great concentration of activated estrogen receptor that competitive binding of this receptor to PPREs of genes regulated by PPARs (Fig. 1 E) may occur, strengthening even more the effects of a lower PPARα expression. It is possible, as well, that estradiol negative regulation of the assayed enzymes catalase and urate oxidase might be due to the same repression mechanism via estrogen receptor blockage of the promoter region of these genes.

Although catalase expression is not known to be directly influenced by PPARα, the seasonal changes observed both in catalase gene expression and enzymatic activity paralleled the seasonal variation of PPARα. Considering the H_2O_2 degrading activity of catalase, expression increases or decreases of several H_2O_2 generating oxidases up-regulated by PPARα may well be the reason for this connection. Conversely, the similarity between urate oxidase activity and PPARα expression seasonal variations in females does not apparently fit with known mechanisms, once the expression of this enzyme is not induced by peroxisome proliferators. This interesting subject may possibly serve as theme for future research.

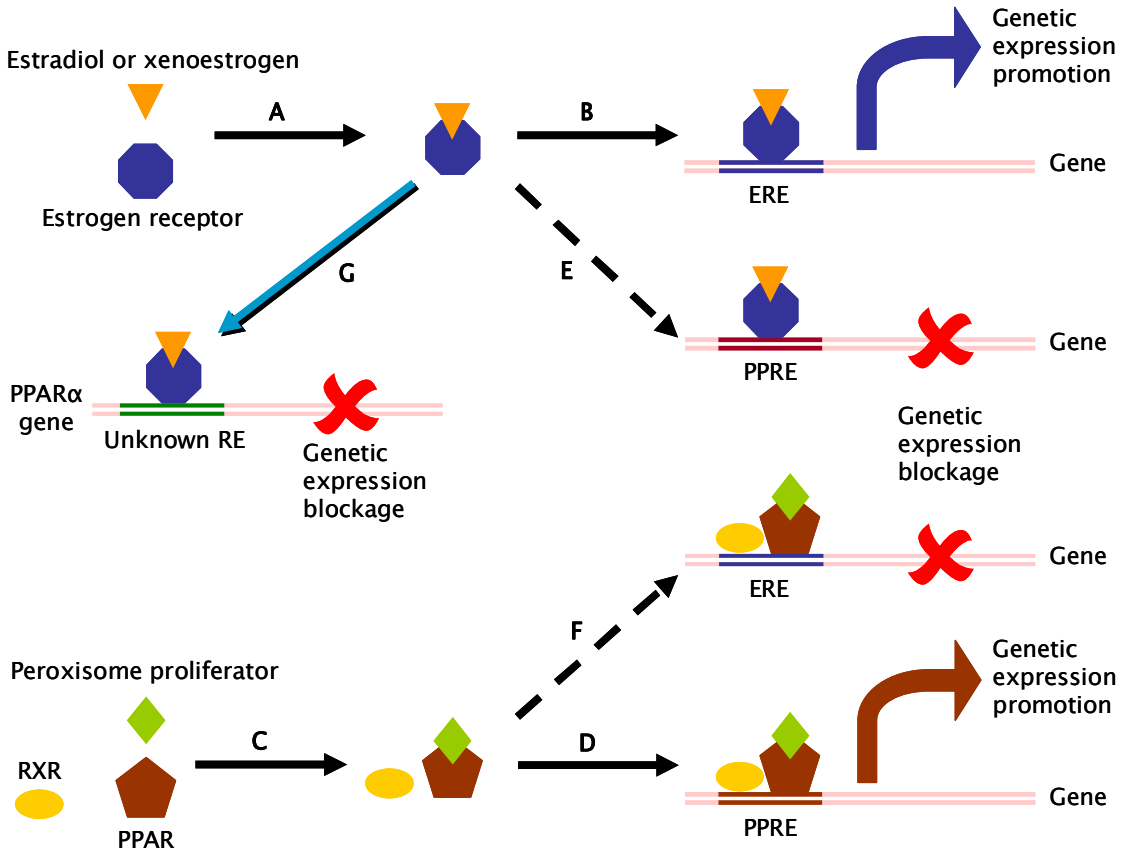

Fig. 1 – Estradiol hypothetical regulation of brown trout hepatic peroxisomes. After estrogen activation (A), the estrogen receptor (homodimerized) is able to bind to an estrogen receptor response element (ERE) and subsequently promote transcription of the corresponding gene (B). Likewise, after peroxisome proliferator activation (C), PPAR heterodimerizes with the retinoid X receptor (RXR) and then the complex is able to bind to a peroxisome proliferator response element (PPRE), subsequently promoting transcription of the corresponding gene (D). When in high concentrations, activated estrogen receptors can competitively bind to a PPRE (E), and, upon activation, PPARs can also competitively bind to an ERE (F), blocking the respective genetic expressions. Finally, the activated estrogen receptor is hypothetically able to bind to an unknown response element (RE) in PPARα gene, thus repressing its transcription (G).

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8. EPILOGUE

Working model of excellence,
She seasonally changes humour
Brown trout was our preference
To distinguish fact from rumour

Sensitive liver was not neglected
He really is one of a kind
In the end, he's always affected
By a hormonal guideline

Estrogens influence expression
Of PPARs and catalase
And cause activity depression
Also on urate oxidase

With peroxisome morphology
They have a calmer interaction
Stereological methodology
Shows a weak and slow reaction

Is the peroxisome under
Estradiol regulation?
With the estrogen receptor
He has an intriguing relation

Complex, somewhat
It must be seen as a whole
"She loves me, she loves me not"
I believe she does, after all…